

THE UNIVERSITY OF CAPE TOWN

NEURON-GLIAL INTERACTIONS IN DENDRITE GROWTH

by

PETER D. LE ROUX

A DISSERTATION SUBMITTED TO THE FACULTY OF MEDICINE IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DOCTOR OF MEDICINE

DEPARTMENT OF NEUROSURGERY

© PETER D. LE ROUX 1994

Paris, France

July 1994

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

DECLARATION**NEURON GLIAL INTERACTIONS IN CORTICAL NEURON DENDRITE
GROWTH.**

I, Peter D. Le Roux hereby declare that the work on which this thesis is based is my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

| |
|---------------------|
| Signed by candidate |
|---------------------|

Signature Removed



PETER D. LE ROUX

29 July 1994

**ABSTRACT: NEURON GLIAL INTERACTIONS IN CORTICAL NEURON
DENDRITE GROWTH.**

Interactions between neurons and glia occupy a central role in many aspects of development, maintenance, and function of the central nervous system (CNS). A fundamental event in CNS development is the elaboration of two distinct neuronal processes, axons and dendrites. The overall aim of this research was to characterize the interactions between central nervous system neurons and astroglial cells that regulate dendrite growth from cerebral cortical neurons.

Embryonic (E18) mouse cerebral cortical neurons were cocultured with early postnatal (P4) rat astroglia derived from cerebral cortex, retina, olfactory bulb, mesencephalon, striatum and spinal cord. Axon and dendrite outgrowth from isolated neurons was quantified using morphological and double-labeling immunohistochemical techniques at 18 hours and 1, 3 and 5 days *in vitro*. Neurons initially extended the same number of neurites, regardless of the source of glial monolayer; however, astroglial cells differed in their ability to maintain primary dendrites. Homotypic cortical astroglia maintained the greatest number of primary dendrites. Astroglia derived from the olfactory bulb and retina maintained intermediate numbers of dendrites, whereas only a small number of primary dendrites were maintained by astroglia derived from striatum, spinal cord or mesencephalon. Initially longer axons were observed from neurons grown on astroglia that did not maintain dendrite number. After 5 days *in vitro*, axon growth was similar on the various monolayers, total primary dendrite outgrowth, however, was nearly threefold greater on astroglia derived from the cortex, retina and olfactory bulb than on astroglia derived from mesencephalon, striatum or spinal

cord. This effect was principally on the number of primary dendrites rather than the elongation of individual dendrites and was independent of neuron survival.

Similar morphological differences were observed after 5 days *in vitro* when cortical neurons were grown on polylysine in either a noncontact coculture system where astroglia continuously conditioned the culture medium or in astroglial conditioned medium. Preliminary biochemical analysis of the medium conditioned by cortical astroglia using heat and trypsin degradation, ultracentrifugation, dialysis, and heparin affinity chromatography suggested that a heparin binding protein with a molecular weight between 10 and 100kDa may be responsible for astroglial mediated dendrite growth. Neurons that were grown in medium conditioned by either mesencephalic or cortical astroglia for the first 24 hours followed by culture medium from astroglia of the alternate source for 4 days *in vitro*, confirmed that astroglia maintained, rather than initiated, the outgrowth of the primary dendritic arbor.

In the next series of experiments, E18 mouse cortical neurons were cocultured with neonatal (P4) or mature (P12) rat astroglia derived from cortex and mesencephalon or astroglia derived from P4 and P12 lesioned cortex. After 5 days *in vitro* the maturational age of astroglia did not appear to alter the extent of primary dendrite growth; instead dendrite growth reflected the region of the CNS from which the astroglia were derived. By contrast, a reduced ability to support axon growth from mouse cortical neurons in culture was observed on astroglia derived from mature rat cortex or mesencephalon. Reactive astroglia demonstrated similar neurite supporting characteristics to mature astroglia and were able to maintain dendrite growth, principally primary dendrite number. Axon elongation, however, was reduced on both neonatal and mature reactive astroglia. Neuron survival did not correlate with the ability of the various astroglia to support process outgrowth.

Collectively these results indicate: 1) neuron-glia interactions are critical for the regulation of process outgrowth from embryonic cortical neurons *in vitro*, 2) axon and dendrite growth appear to be differently controlled by astroglia, 3) CNS astroglia demonstrate regional differences in maintaining, but not initiating growth of the primary dendritic arbor, 4) this effect may be due, in part, to release of a diffusible heparin binding protein factor, and 5) mature and reactive astroglia support primary dendrite, but limited axon growth.

We propose therefore that the local astroglial environment maintains primary dendrite growth from neurons until synaptic contacts can be established. A mechanism that maintains the primary dendritic arbor and allows separate regulation of axon and dendrite growth, prior to the arrival of afferents, may be critical for establishing appropriate and specific synaptic connections. These findings have important implications in understanding development and function of the mammalian central nervous system and may lead to novel strategies for intervention in acute and chronic neurological disorders.

Key words: Astrocyte, astroglia, axon, cortex, dendrite, development, glia, gliosis, injury, neuron, neurite.

ACKNOWLEDGMENTS

The work which culminated in this thesis was performed in the laboratories of Dr. Thomas A. Reh, Department of Biologic Structure, University of Washington, Seattle, WA, U.S.A. and Dr. Alain Prochiantz, Ecole Normale Supérieure, Paris, France between July 1991 and May 1994. I am deeply indebted to their support, inspiration, guidance and thoughtful discussions at all stages in completing this thesis.

This work was supported, in part, by a National Institute of Health grant to TAR and by awards from the Brain Trauma Foundation and New York Academy of Medicine to PLR. I gratefully acknowledge the financial support from these organizations.

I thank Macie Walker, Kirsten Whiting, Roger Williams, and Mark Morris for technical assistance and advice; Ivar Klajvin, Raymond Anchan, Catrin Pittack, Chris Jasoni, and Matt Kelley for help and advice throughout this study and for useful comments on the manuscript. I would also like to thank Renee Czarapata for compiling the references and Paul Schwarz and Janet Claredy for excellent photographic assistance.

I wish to sincerely thank my parents, Peter and Sally, for laying the foundation, Professor J.C. de Villiers and Professor Frances Ames for stimulating an early interest in the Neurosciences, Professor H. Richard Winn for inspiration, advice and support and Eleanor for her love, understanding and encouragement; this thesis would not have been possible without their contributions.

CONTENTS

| | |
|------------------------|-----------|
| Declaration | <i>I</i> |
| Abstract | <i>II</i> |
| Acknowledgments | <i>V</i> |

SECTION 1: BACKGROUND

| | | |
|----------|--|----|
| 1 | INTRODUCTION AND SPECIFIC AIMS | 1 |
| 2 | DEVELOPMENTAL NEUROBIOLOGY: THE LINK BETWEEN SCIENTIST AND CLINICIAN. | 4 |
| 3 | NEURONS ELABORATE TWO TYPES OF PROCESSES: AXONS AND DENDRITES. | 7 |
| | Introduction | 7 |
| | Neurons elaborate two different types of processes: axons and dendrites | 7 |
| | Morphology | 8 |
| | Organelles and biochemistry | 9 |
| | Surface structure | 10 |
| | Cytoskeletal composition | 11 |
| | Microtubule polarity | 12 |
| | Synaptic structure | 12 |
| | Ion channels, electrophysiology and function | 14 |
| | Growth characteristics | 14 |
| | Sorting of axonal and dendritic constituents | 15 |
| 4 | DENDRITES: PHYSIOLOGIC AND PATHOLOGIC SIGNIFICANCE. | 16 |
| | Introduction | 16 |
| | Dendrites influence synaptic function | 16 |
| | Dendrites and neural plasticity | 17 |
| | Dendrites, plasticity and neuronal cell loss | 18 |
| | Dendrites and acute neurologic insults | 18 |
| | The dendritic cytoskeleton and acute neurologic insults | 19 |
| | Dendrites and neurodegenerative conditions | 21 |
| 5 | DENDRITE DEVELOPMENT | 24 |
| | Introduction | 24 |

| | |
|--|----|
| Dendritic ontogeny recapitulates phylogeny | 26 |
| Genes and dendrite growth | 26 |
| Intrinsic control of dendrite growth: arguments for and against | 27 |
| Dendrite growth and the cytoskeleton | 29 |
| Dendrite growth: the role of afferents, synapses and neurotransmitters | 31 |
| Can dendrite growth occur without afferent or synaptic influence? | 33 |
| Neuron activity determines the final form of the dendritic arbor | 34 |
| Glial cells influence dendrite growth | 36 |
| The role of nutrition, hormones and opioids on dendrite growth | 38 |
| Towards a theory of dendrite development | 40 |

SECTION 2: EXPERIMENTS

| | |
|---|----|
| 6 METHODOLOGY | 42 |
| Glial cells | 42 |
| Neurons | 46 |
| 7 MATERIALS AND METHODS | 52 |
| Tissue culture | 52 |
| Dissociated Cerebral Cortex Neuronal Cultures | 52 |
| Glial cell cultures | 53 |
| Mature and reactive glial cell cultures | 54 |
| Non contact transfilter experiments | 55 |
| Conditioned medium | 55 |
| Protein biochemistry | 56 |
| SDS-PAGE and immunoblotting of mouse cortex | 56 |
| Denaturation of conditioned medium | 56 |
| Protein concentration | 57 |
| Heparin affinity chromatography | 57 |
| Protein radiolabeling | 58 |
| Protein extraction from glial monolayers and conditioned medium | 58 |
| Peptide growth factors | 59 |
| Immunohistochemistry and analysis | 59 |
| Immunohistochemical identification of cells in culture | 59 |
| Analysis of cortical neuron survival and process outgrowth | 61 |

| | |
|---|-----------|
| 8 THE DEVELOPMENTAL TIME COURSE OF PROCESS SPECIFIC CYTOSKELETAL PROTEIN EXPRESSION IN EMBRYONIC MOUSE CORTICAL NEURONS <i>IN VITRO</i>. | 64 |
| Introduction | 64 |
| Materials and methods | 66 |
| Results | 66 |
| Cytoskeletal and surface antigen recognition in E18 mouse cortical neurons | 66 |
| Neurite identification | 67 |
| Morphology of developing axons and dendrites | 70 |
| Distribution of cytoskeletal proteins in developing mouse cortical neurons | 71 |
| Discussion | 78 |
| MAP2 and NF-H expression in developing mouse cortical neurons | 83 |
| Synthesis and assembly of cytoskeletal proteins | 85 |
| Extrinsic signals and the cytoskeleton | 87 |
| 9 REGIONAL DIFFERENCES IN ASTROGLIAL DERIVED FACTORS THAT PROMOTE DENDRITIC OUTGROWTH FROM MOUSE CORTICAL NEURONS <i>IN VITRO</i>. | 89 |
| Introduction | 89 |
| Materials and methods | 94 |
| Results | 95 |
| Glial Characterization | 95 |
| Dendritic growth is selectively influenced by the anatomic source of the astroglial monolayer | 102 |
| Axon length does not differ on astroglial cells from diverse CNS regions | 110 |
| Diffusible factors released by astroglia may account for part of the regional differences observed in dendrite growth | 111 |
| Analysis of neuron survival and morphology | 113 |
| Discussion | 118 |
| Astroglia influence the number of primary dendrites | 119 |
| Astroglial origin does not determine axon length | 121 |
| Astroglial heterogeneity | 123 |
| Mechanism of astroglial mediated dendrite growth | 125 |

| | | |
|-----------|---|------------|
| 10 | ASTROGLIA DEMONSTRATE REGIONAL DIFFERENCES IN THEIR ABILITY TO MAINTAIN PRIMARY DENDRITIC OUTGROWTH FROM MOUSE CORTICAL NEURONS <i>IN VITRO</i>. | 128 |
| | Introduction | 128 |
| | Materials and methods | 130 |
| | Results | 130 |
| | Changes in dendrite number during neuron development | 130 |
| | Axon growth | 139 |
| | Astroglia are necessary to maintain the primary dendritic arbor | 140 |
| | Discussion | 144 |
| 11 | REACTIVE AND MATURE ASTROGLIA SUPPORT PRIMARY DENDRITIC BUT NOT AXONAL OUTGROWTH FROM MOUSE CORTICAL NEURONS <i>IN VITRO</i>. | 149 |
| | Introduction | 149 |
| | Materials and methods | 151 |
| | Results | 151 |
| | Characterization of cellular monolayers | 151 |
| | Dendrite growth is supported by immature and mature astroglia | 154 |
| | Mature astroglia exhibit a reduced capacity to support axon growth | 154 |
| | Reactive cortical astroglia demonstrate a reduced ability to support axon growth but not dendrite number | 159 |
| | Reactive astroglia may release diffusible factors that support neurite growth | 160 |
| | Fibroblast influence on astroglial supported axon and dendrite growth | 165 |
| | Neuron survival | 168 |
| | Discussion | 175 |
| | Mature astroglia support dendrite growth but demonstrate a reduced ability to support axon growth | 175 |
| | Reactive astroglia in the immature and mature mammalian brain | 176 |
| | Reactive astroglia support primary dendrite number but not axon growth | 177 |
| | What accounts for limited growth of axons observed on reactive astroglia? | 178 |

| | |
|--|----------------|
| 12 THE MOLECULAR BASIS OF DENDRITE GROWTH: A PRELIMINARY ANALYSIS OF ASTROGLIAL CONDITIONED MEDIUM AND REVIEW OF POTENTIAL MECHANISMS OF ASTROGLIAL MEDIATED DENDRITE GROWTH. | 183 |
| Introduction | 183 |
| Materials and methods | 184 |
| Results | 185 |
| Neurite outgrowth in astroglial conditioned medium | 185 |
| Protein factors mediate neurite growth | 187 |
| Astroglial conditioned medium derived from various CNS sources contain different proteins | 192 |
| Factors between 10kDa and 100kDa support dendrite growth | 192 |
| Fibroblast growth factor supports dendrite and axon growth | 195 |
| Fibroblast growth factor is probably present in cortical astroglial conditioned medium | 199 |
| Discussion | 199 |
| Astroglial conditioned medium and dendrite growth | 200 |
| Peptide growth factors and dendrite growth | 203 |
| Fibroblast growth factor | 204 |
| The extracellular matrix, proteoglycans and dendrite growth | 206 |
| Cell adhesion and dendrite growth | 209 |
| Molecular diversity and synergism | 212 |
| <u>CONCLUDING REMARKS</u> | 215 |
| <u>LITERATURE CITED</u> | 222 |

INTRODUCTION AND SPECIFIC AIMS

The elaboration of two distinct neuronal processes, axons and dendrites is a fundamental event in neuron differentiation and of critical significance as it establishes the neuron's functional polarity in the nervous system (Kuffler et al 1984, Steward 1989a, Kandel et al 1991, Peters et al 1991, Spruston et al 1994). Despite the enormous diversity of dendritic form in the mammalian central nervous system (CNS; Ramon y Cajal 1954, Peters et al 1991), little is known about the precise mechanisms that regulate establishment and maintenance of dendrite morphology.

An understanding of the factors involved in the growth and maintenance of the dendritic arbor of CNS neurons will substantially impact both the basic and clinical neurosciences. First, dendritic remodeling and growth occurs throughout life (Greenough et al 1985, Levine and Truman 1985, Coleman and Flood 1986, Purves et al 1986, Petit et al 1988, Purves et al 1988, Black et al 1990, Kolb and Gibb 1991, Popov et al 1992, Jones and Schallert 1994) and is thought to underlie memory (Lynch et al 1986, Schacher et al 1993), learning (Greenough et al 1985, Black et al 1990) and adaptation to the environment (Feldman and Dowd 1975, Coleman and Flood 1976, Greenough et al 1979, Purves et al 1986, 1988, Jones and Schallert 1994). Second, abnormalities in dendrite structure are frequently observed in acute neurologic insults such as trauma or stroke (Mattson et al 1988a, Kitigawa et al 1989, Taft et al 1992) and in neurodegenerative conditions such as Alzheimer's disease (Buell and Coleman 1979, McKee et al 1989). Finally, following injury dendrite growth is thought to play a role in recovery of neuron function (Coleman and Flood 1986, Jones and Schallert 1994).

Over the past 15 years it has become increasingly apparent that glial cells are important to many aspects of neuron growth and differentiation during development of the CNS. Following neuron generation in the ventricular and subventricular zone of the embryonic brain, radial glia guide neuroblasts to their appropriate location (Levitt and Rakic 1980, Rakic 1988, Snow et al 1990a and 1990b, Steindler 1993). The pioneering axons in the long fiber tracts of the CNS typically grow in association with the end feet of radial glia (Silver et al 1982, Silver and Ogawa 1983, Norris and Kalil 1991) while *in vitro* studies have consistently found that astroglia will support neuron survival (Banker 1980) and neurite outgrowth (Noble et al 1984, Fallon et al 1985, Tomaselli et al 1988). More recently tissue culture experiments have demonstrated that astroglia can influence neuron polarity and dendrite morphology in sympathetic (Bruckenstein and Higgins 1988, Tropea et al 1988, Johnson et al 1989), sensory (De Koninck et al 1993), and mesencephalic neurons (Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Rousselet et al 1988 and 1990).

Whether astroglia can influence the growth of dendrites from cerebral cortical neurons is not known. Furthermore, the characteristics of astroglial mediated dendrite growth are largely unknown. Therefore, the overall aim of this research was to characterize the interactions between CNS neurons and glial cells that regulate cerebral cortical neuron dendrite growth. The specific aims addressed by the experiments described in this report were as follows:

1. Do astroglia influence dendrite growth from cerebral cortical neurons?

2. Do astroglia derived from various CNS regions demonstrate regional differences in supporting dendrite growth from cortical neurons?
3. Do astroglia mediate dendrite growth by cell-specific interactions or by the release of diffusible factors?
4. Do astroglia initiate or maintain growth of the dendritic arbor from cortical neurons?
5. Do mature astroglia support dendrite growth?
6. Do reactive astroglia support dendritic growth?
7. Do astroglia release an identifiable factor or factors that support dendrite growth?

DEVELOPMENTAL NEUROBIOLOGY: THE LINK BETWEEN SCIENTIST AND CLINICIAN

Neurologic injuries and disease that lead to neuron loss or degeneration represent a significant health problem. For example, in the United States spinal cord injuries leading to paralysis affect 10, 000 people a year, head injuries about 500, 000, stroke about 1.5 million, and neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, and Huntington's disease affect millions more (Kurtzke 1975, Caveness 1979, Kalsbeek et al 1980, Kurtzke 1983, Terry and Katzman 1983, Dyken et al 1984, AHA, Stroke Facts 1986). Many patients afflicted by these conditions now survive as a result of improved health care and advances in diagnosis. Prognosis for full neurologic recovery has generally been poor; however, knowledge recently gained from basic science research suggests that a real hope of recovery may soon be possible.

Experimental evidence accumulated in the last 20 years demonstrating reorganization and growth of the CNS following injury in both animals and humans (Raisman 1969, Grady et al 1989), has challenged the prevailing notion that the mammalian CNS does not regenerate. Furthermore, it is now apparent that reorganization of the CNS occurs in a wide variety of settings, appears to be functionally significant, and may contribute to behavioral recovery (Steward 1989b). This discovery of CNS plasticity has opened the door for therapies that potentially can modify outcome after CNS injury.

Until recently there have been few direct treatments for CNS-injured patients; most efforts have been directed toward stabilizing the physiologic

condition, preventing secondary complications, or rehabilitation. During the last ten years, however, treatments aimed at preventing or reversing the damage of CNS injury and promoting repair of the tissue have emerged. The majority of these emerging treatments including: 1) administration of exogenous substances that prevent the cascade of deterioration resulting from loss of neuronal trophic support or that stimulate and promote naturally occurring growth responses (Kromer 1986, Walicke et al 1986, Morrison et al 1987, Uniscker et al 1987, Yamada et al 1991, Gluckman et al 1992, Mattson et al 1993, Thanos et al 1993, Svedsen et al 1994), 2) replacement of lost tissue by grafts (Villegas-Pérez et al 1988), 3) insertion of implantable drug infusion pumps, polymer drug delivery systems, biocompatible mechanical nerve guides, or sensory prostheses (Harbaugh 1989, Lipton 1989), and 4) neural transplantation using fetal, adrenal, neural, glial, genetically engineered, or immortalized cells (Björklund and Steneveit 1984, Sladek and Gage 1988, Klot et al 1990, Blakemore and Franklin 1991, Kawaja and Gage 1991, Frim et al 1993), have been observed to stimulate neuron survival, growth and differentiation *in vitro* or promote functional recovery in experimental animals. In humans, however, use of these various therapeutic strategies are still in their infancy (Lindvall 1991, Madrazo et al 1991).

Potential therapeutic approaches have evolved because of improved experimental techniques by which complex structures can be analyzed and reduced to their constituent parts; such a reductionist approach has resulted in advances in understanding basic cellular and molecular properties of the CNS (Jacobsen 1991). Of importance to neurosurgeons and neurologists is the close relationship between development, degeneration and regeneration (Holder and Clark 1988, Bastmeyer et al 1993). Numerous factors that promote

survival or differentiation of developing CNS neurons can also prevent cell death in a variety of conditions and so provide a promise of effectively treating acute injuries, stroke or neurodegenerative conditions (Sugiyama et al 1989, Mattson and Rychlik 1990, Cheng and Mattson 1991, Rosenberg 1991, Yamada et al 1991, Mattson et al 1993, Svendsen et al 1994). Factors involved in development may or may not mediate regeneration, however, the study of neurodevelopment is critical if we are ultimately to devise rational therapeutic strategies to reverse the clinical and behavioral manifestations of brain injury, cerebral ischemia, or neurodegenerative conditions.

NEURONS ELABORATE TWO TYPES OF PROCESSES: AXONS AND DENDRITES:

Introduction.

The adult mammalian brain is made of individual units: neurons and glia. The complex and diverse functions of the mammalian CNS including perception, memory, coordination, and motor movement depend on specific connections and interactions between neurons, neurons and glia, and between neurons and end organs. In large part, the particular pattern of connections is determined by the morphology and structure of the component parts (Lasek 1988, Kandel et al 1991, Schacher et al 1993).

Neuron structure was not appreciated until the late 19th century when Camillo Golgi (1873) introduced a histological technique, silver impregnation, that allowed individual neurons to be seen. Using this technique, Ramon y Cajal (1894 and 1954) was able to describe the fine structure of the nervous system, including neuron structure, differences between neurons, and the precise connections between neurons. What Cajal and neuroanatomists since then have observed is that the feature that most dramatically distinguishes neurons is their shape, specifically the number and form of processes elaborated by the cell (Nauta and Freitag 1984, Steward 1989a, Kandel et al 1991, Peters et al 1991).

Neurons elaborate two different type of processes: axons and dendrites.

Multipolar neurons predominate in the mammalian central nervous system. Each neuron varies in size and shape, but each exhibits a complex form,

highly characteristic of that neuron type, that is largely determined by the morphology of its processes. Well differentiated, mature neurons elaborate two types of processes *in vitro* and *in vivo*. The two types of processes, axons and dendrites differ from each other in: 1) morphology (Peters et al 1991, Steward 1989a, Deitch and Banker 1993), 2) organelles and biochemistry (Torres and Steward 1992, Deitch and Banker 1993), 3) surface structure (Craig et al 1992, Ferreira et al 1993), 4) cytoskeletal composition (Caceres et al 1986, Kosik and Finch 1987, Pennypacker et al 1991), 5) microtubule polarity (Baas et al 1988, Craig et al 1992), 6) synaptic structure (Bartlett and Banker 1984b, Fletcher et al 1991), 7) ion channels, electrophysiology and function (Steward 1989a, Kandel et al 1991, Craig et al 1993, Spruston et al 1994), and 8) growth characteristics (Dotti et al 1988, Sargent 1989).

Morphology.

Neurons typically elaborate one axon that arises from a specialized region of the neuron cell body, the axon hillock. By contrast, a neuron typically has several dendrites, that branch out from the cell body in a tree like manner (Barlett and Banker 1984a and 1984b, Peters et al 1991, Deitch and Banker 1993). Axons are long, thin and of relatively uniform diameter until their terminal arborization, whereas dendrites are relatively short, tapering processes, whose length is proportional to the diameter of their stem (Dotti et al 1988). In addition, axons may be myelinated and can cross the interface between the peripheral and central nervous systems; dendrites, however, are very rarely myelinated and generally do not cross the peripheral and central nervous system interface. Finally, the branching pattern differs: axons tend to elaborate branches at right angles depending on their interaction with their surroundings, whereas dendrite branching can occur at a variety of angles.

Organelles and biochemistry.

Dendrites are in many ways extensions of the cell body. Ultrastructural and *in situ* hybridization studies reveal that dendrites contain polyribosomes and other organelles including rough endoplasmic reticulum and golgi bodies, whereas axons have a greater density of smooth endoplasmic reticulum, particularly within the axonal growth cone (Bartlett and Banker 1984a and 1984b, Steward 1989a, Peters et al 1991, Torres and Steward 1992, Deitch and Banker 1993). The paucity of ribosomes in axons is one of most reliable means of differentiating axon and dendrite.

The fundamental biochemical difference between axons and dendrites is the capability for protein synthesis (Torres and Steward 1992). In mature neurons protein synthetic machinery is restricted to the cell body and dendrites, whereas the axon hillock and axon cannot synthesize proteins. Proteins required for maintenance of axonal integrity and synaptic transmission must be carried distally by axonal transport.

Several lines of evidence indicate that local protein synthesis occurs in dendrites. First, dendritic polyribosomes are often found under sites of synaptic specialization and become prominent during synapse growth (Bartlett and Banker 1984b, Steward 1989a, Peters et al 1991, Torres and Steward 1992, Deitch and Banker 1993). Second, a culture technique that allows axons and dendrites to be cultured separately, demonstrates that labeled amino acids are incorporated into dendrites, but not axons (Torres and Steward 1992). Finally, following ^3H -uridine pulse labeling, recently synthesized RNA is selectively transported into dendrites of hippocampal

neurons in culture (Davis et al 1987). Most of the mRNA is for MAP2 and calcium/calmodulin dependent protein kinase, two proteins integral to cytoskeletal stability (Kleinman et al 1990). Taken together, these observations suggest that local protein synthesis may induce modifications of synaptic structure, which is consistent with observations demonstrating that dendrites are sites of the highest oxidative energy metabolism in the nerve cell, particularly during periods of synaptogenesis (Wong-Riley 1989).

Surface structure.

Axons and dendrites differ in their complement of proteins, receptors, and adhesion molecules located on their surface membranes (Craig et al 1992). For example, in hippocampal neurons in culture the receptors for α -bungarotoxin (Banker and Waxman 1988), or transferrin (Cameron et al 1991) are restricted to the somatodendritic domain.

The selective distribution of membrane proteins correlates with the development of neuronal polarity. For example, immunoreactivity for the membrane associated protein, GAP-43, is initially uniformly distributed in developing hippocampal neurons in culture. At the earliest stage an axon can be identified, GAP-43 is preferentially localized to the axon growth cone, whereas other processes that develop into dendrites lose GAP-43 immunoreactivity (Goslin and Banker 1990, Goslin et al 1990).

Membrane spanning glycoproteins present on axons and dendrites differ. For example, amyloid precursor protein (APP) immunoreactivity is highly enriched in the axonal compartment of hippocampal neurons in culture (Ferreira et al 1993). The axonal localization of APP is consistent with its

recently identified role in axon elongation (Sola et al 1993, Moya et al 1994). Dendrites do not demonstrate immunoreactivity for APP, however, the mRNA for APP has been reported in dendrites of neurons in culture (Strong et al 1990). By contrast, tissue section studies do not suggest a dendritic localization for APP mRNA (Lewis et al 1988).

Membrane anchored proteins may be selectively distributed to axons or dendrites. For example, in hippocampal neurons *in vitro*, neuronal glycosylphosphatidylinositol (GPI) anchored Thy-1 is localized to the axon surface (Dotti et al 1991). Another GPI anchored protein, TAG-1, a cell adhesion molecule, is also localized to axons (Furley et al 1990). Not all GPI anchored proteins, however, are located exclusively in axons. For example, F3/F11 is found in both axons and dendrites (Craig et al 1992).

Cytoskeletal composition.

The neuronal cytoskeleton is composed of several classes of molecules, such as microfilaments, intermediate filaments, microtubules and microtubule associated proteins (Cleveland and Hoffman 1991, Solomon 1992). Immunohistochemical studies both *in vitro* and *in vivo* demonstrate that molecular differences between axons and dendrites extend to the cytoskeleton (Shaw et al 1985, Dotti et al 1987, Kosik and Finch 1987, Pennypacker et al 1991). For example, neurofilament, an intermediate filament, exists in several isoforms of different molecular weights in axons and proximal dendrites; the phosphorylated, high molecular weight isoform (NF-H), however, is only found in fully differentiated axons (Sternberger and Sternberger 1983, Shaw et al 1985, Dahl et al 1986, Pennypacker et al 1991).

The structural architecture of the cytoskeleton differs in axons and dendrites. For example, in mature axons, microtubules, composed of tubulin, are arranged in tightly packed bundles, whereas they are more widely spaced in dendrites (Bartlett and Banker 1984a and 1984b). In part, microtubule spacing is determined by the presence of a particular microtubule associated protein (Chen et al 1992).

Microtubule associated proteins (MAP's) are a class of unique accessory proteins that bind and promote the assembly of neuronal microtubules (Cleveland and Hoffman 1991, Lee 1993); they are selectively localized in differentiated neurons. For example, MAP2, a high molecular weight, heat stable protein is found in dendrites (Cáceres et al 1984a, 1984b and 1986, Matus et al 1986, Dotti et al 1987). By contrast, axons express a low molecular weight MAP, tau (Kosik and Finch 1987, Lee 1993). Other microtubule associated proteins, such as MAP1b, are selectively phosphorylated in axons.

Microtubule polarity.

Microtubules in the axon are all oriented in the same direction; the plus end is oriented toward the direction of the growth cone, whereas the minus end is anchored in the microtubule organizing center located in the cell body. By contrast, microtubules in dendrites extend their plus ends toward or away from the cell body (Baas et al 1988, Craig et al 1992).

Synaptic structure.

Synapses are a unique characteristic of neurons; axons and dendrites, however, differ in their contribution to synaptic structure and polarity. Axons terminate in presynaptic terminals, whereas dendrites are generally

postsynaptic (Bloom 1972, Bartlett and Banker 1984a and 1984b, Peters et al 1991). Exceptions, such as axo-axonic or dendro-dendritic synapses are found in the olfactory bulb and retina.

Axons and dendrites demonstrate several distinctive structural differences at the synapse. For example, dendritic microspecializations known as dendritic spines are found at sites of synaptic contact. The size and length of dendritic spines varies with the activity of the presynaptic terminal (Schade et al 1972, Globus et al 1973, Feldman and Dowd 1975, Purpura 1975, Harris and Stevens 1988). Dendritic spines, however, are difficult to evaluate as they are near the limit of light microscopic resolution; the exact function, therefore, is not known. Experimental and theoretical evidence is now accumulating that dendritic spines create an isolated biochemical environment around synapses and influence the dynamics of intracellular second messengers such as calcium (Koch and Zador 1993).

Synaptic vesicle antigens are selectively distributed to axons (Fletcher et al 1991), consistent with axon storage and release of neurotransmitters (Young and Poo 1983). By contrast, use of *in situ* hybridization and immunohistochemical techniques suggests that some growth factors, such as brain derived neurotrophic factor (BDNF), may be selectively released from the dendritic compartment of rat hippocampal and cortical neurons in culture (Wetmore et al 1991 and 1994). This selective release of growth factors may play a potential autocrine or paracrine role and provide local trophic support for the maintenance of plasticity and reorganization in the CNS (Mattson et al 1989 and 1993).

Ion channels, electrophysiology and function.

The dendritic compartment contains various neurotransmitter gated ion channels that determine whether a synapse is excitatory or inhibitory. For example, glycine, gamma aminobutyric acid (GABA, Killisch et al 1991), and selective glutamate (alpha-amino 3-hydroxy-5-methyl-4-isoxaozole proprionic acid, AMPA, Craig et al 1993) gated ion channels are found on dendrites. By contrast, axons express sodium channels (Kandel et al 1991).

The axon is the main conducting unit of the neuron, whereas dendrites are the principle receptive surface of the neuron (Kuffler et al 1984, Peters et al 1991). Axons propagate an all or none transient electrical signal, the action potential, over great distances. The action potential is based on transient opening of voltage dependent sodium channels that generate membrane depolarization followed by repolarization that is mediated by increased potassium conductance. Once an action potential is initiated in any part of the axon, it is conducted on unless there is a physical interruption in the axon, or appropriate ion channels are inactivated. Most dendrites conduct passively as cable conductors; dendrite shape, in large part, determines the conducting properties (Steward 1989a, Kandel et al 1991). The discovery of voltage-gated ion channels on dendrites, however, is now challenging this view (Spruston et al 1994).

Growth characteristics.

Axons and dendrites demonstrate different growth characteristics (Dotti et al 1988, Sargent 1989). The neuron first supports growth of a single axon, then growth of multiple dendrites. In general, axons elongate faster, grow long distances, fasciculate, and arborize when they reach their target. Dendrites

grow shorter distances, do not fasciculate and arborize locally. The differences in growth characteristics implies that the development of axons and dendrites may be differently regulated; the growth and elaboration of dendrites is reviewed more fully in chapter 5.

Sorting of axonal and dendritic constituents.

How axonal and dendritic constituents are segregated into polarized domains is not clear (Craig et al 1992). Recent experimental observations, however, suggest the interaction of both intrinsic and extrinsic signaling mechanisms. For example, synaptic vesicle proteins are selectively distributed in isolated hippocampal neurons *in vitro*; the formation of large clusters typical of presynaptic specializations, however, requires contact with the appropriate target (Fletcher et al 1991).

Sorting of neuronal surface components may also involve an interaction of mechanisms intrinsic to individual neurons and mechanisms mediated by cell-cell interactions. For example, the viral glycoproteins, vesicular stomatitis virus G protein and fowl plague virus hemagglutinin are sorted to the axonal and somatodendritic domains respectively, in cultured hippocampal neurons (Dotti and Simons 1990). In epithelial cells these same viral glycoproteins are sorted to the apical and basolateral domains. The onset of polarity in epithelial cells requires an internal mechanism of vesicular sorting, but is only initiated in response to external signals such as cell-substrate and cell-cell adhesion (Simons et al 1992).

DENDRITES: PHYSIOLOGIC AND PATHOLOGIC SIGNIFICANCE

Introduction.

The dendritic arbor of CNS neurons can influence the overall function of the CNS in several ways. First, dendritic variation is one of the most striking features of CNS nerve cells (Ramon y Cajal 1954, Peters et al 1991) and of critical significance as it establishes polarity and allows each neuron to function in a network to receive, process, store, and transmit information. Second, dendrites appear to be among the most plastic morphologic elements in the nervous system (Purves et al 1988), and may compensate for neuronal loss in the CNS that accompanies normal aging (Coleman and Flood 1986, Jones and Schallert 1994). Finally, although the nature of the dendritic response to injury has not been clearly established, data is accumulating from many laboratories that implicates dendrites in several pathologic states ranging from Alzheimer's disease (Buell and Coleman 1979, McKee et al 1989) to stroke (Mattson et al 1988a, Kitigawa et al 1989, Shigeno et al 1993) and trauma (Taft et al 1992). This chapter will briefly review the physiological and pathological significance of the dendritic arbor.

Dendrites influence synaptic function.

The intimate pattern of synaptic connections in the CNS underlies all aspects of neuronal function (Kuffler et al 1984, Spruston et al 1994); these interactions are, in part, regulated by dendritic structure (Midtgaard 1994). Input from different presynaptic neurons is often distributed to several dendritic compartments at the synapse. The spatial and temporal integration of signals from various presynaptic neurons depends on the electrical properties of the dendrites forming the synapse and the distance between

dendrites; these properties are related to dendrite morphology (Midtgaard 1994). For example, most dendrites act as cable conductors (Steward 1989a); cable conducting properties depend upon the relationship between internal resistance and membrane resistance. Internal resistance is determined by the internal diameter of individual dendrites (Kandel et al 1991).

Dendrites and neural plasticity.

At the end of the 19th century it was postulated that dendrites change shape, retract, or extend in response to functional demands of the environment (Rabla-Rückard 1890, Ramon y Cajal 1894 and 1954). Subsequent experimental data from several systems indicates that the mammalian brain can modify its neuronal circuitry through changes in the number, type and efficiency of synapses primarily by alterations in dendrite number and morphology (Greenough et al 1985, Levine and Truman 1985, Coleman and Flood 1986, Purves et al 1986, Petit et al 1988, Purves et al 1988, Black et al 1990, Kolb and Gibb 1991, Popov et al 1992, Jones and Schallert 1994). For example, quantitative Golgi analysis of hippocampal pyramidal neurons in the hibernating ground squirrel, demonstrates morphologic correlates in dendrite length, branching, and spine morphology with torpor and arousal (Popov et al 1992).

Modification of the dendritic architecture provides a means by which the nervous system can adjust aspects of its functions to age, development and the environment (Feldman and Dowd 1975, Coleman and Flood 1976, Greenough et al 1979, Purves et al 1986 and 1988, Jones and Schallert 1994). Furthermore, it is postulated that these structural changes underlie memory and learning (Lynch 1986, Schacher et al 1993). For example, extensive

exposure to motor learning tasks enhances dendritic arborization and increases synapse number in the cortex and cerebellum (Greenough et al 1985, Black et al 1990). Adults and embryos may use common mechanisms and molecules to modify synapses and dendritic architecture, thereby linking the once separate fields of developmental neurobiology and learning and memory (Cline and Constatine-Paton 1989).

Dendrites, plasticity and neuronal cell loss.

Dendritic proliferation is seen in regions of the brain that demonstrate age related neuron loss (Coleman and Flood 1986). Neuron cell loss is a characteristic feature of many pathologic conditions in the CNS including hypoxia, ischemia, seizures, trauma, and neurodegenerative conditions such as Alzheimer's disease. Generally, neuron cell loss must be substantial before functional deficits are observed suggesting a considerable capacity for neural plasticity. This process of reorganization is poorly understood; one possible mechanism may be dendrite growth (Coleman and Flood 1986, Isacson and Sofroniew 1992, Jones and Schallert 1994). For example, following unilateral lesions to the sensorimotor cortex in experimental animals, significant dendrite growth is observed in the non-lesioned homotopic cortex (Isacson and Sofroniew 1992, Jones and Schallert 1994). The extent of compensatory dendrite growth correlates with the degree of functional recovery (Jones and Schallert 1994).

Dendrites and acute neurologic insults.

Excitatory amino acids (EAA) normally function as neurotransmitters; glutamate, an EAA, excites virtually all CNS neurons. There is substantial evidence, however, that in high concentrations excitatory amino acids,

particularly glutamate, may be integral to the pathophysiology of neuron injury and death in acute events such as epilepsy, stroke or trauma (Tecoma et al 1989, Katayama et al 1990, Beal 1992, Bullock et al 1992, Hayes et al 1992, Baker et al 1993, Lipton and Rosenberg 1994). The cytotoxic effects of glutamate are primarily mediated through agonist action at the N-methyl D aspartate (NMDA) subclass of glutamate receptors and result from excess influx of calcium into cells (Choi et al 1987, Choi et al 1988, Mattson et al 1989, Hartley et al 1993).

Several lines of evidence suggest that dendrites may be particularly vulnerable to excitatory amino acid neurotoxicity. 1) Excitatory synapses are found primarily on dendrites (Peters et al 1991), hence dendrites would be most vulnerable and sensitive to stimulation by excitatory agonists. 2) *In situ* hybridization and immunocytochemistry of mature rat hippocampal neurons in culture demonstrate subcellular localization of glutamate receptors; the NMDA ionotropic receptors Glu R1-3 are localized on dendritic spines (Craig et al 1993). By contrast, axon staining of the glutamate receptor subunit Glu R1 is not observed. 3) Following exposure to toxic amounts of glutamate, or related EAA *in vivo* and *in vitro*, the first neuronal changes observed are acute swelling of dendrites and cell bodies. This is followed by slowly evolving neuronal degeneration. By contrast, axons and glia are relatively refractory to EAA induced lesions (Coyle et al 1981, Schwartz et al 1983, Choi et al 1987, Choi 1988, Rosenberg et al 1999, Shigeno et al 1993).

The dendritic cytoskeleton and acute neurologic insults.

A feature of both ischemia and traumatic brain injury is an increase in extracellular potassium and intracellular calcium (Katayama et al 1990, Siesjö

1992); both can alter the phosphorylation state of MAP2, a component of the cytoskeleton unique to dendrites (Halpain and Greengard 1990, Diaz-Nido et al 1993, Montoro et al 1993). In addition, increased intracellular calcium activates proteases; calcium activated proteases and the phosphorylation state of MAP2 play critical roles in the normal processing of cytoskeletal proteins (Tsuyama et al 1987, Yamauchi and Fujisawa 1988, Johnson et al 1991). For example, highly phosphorylated MAP2 is unable to bind to tubulin (Tsuyama et al 1987) or promote actin bundling (Yamauchi and Fujisawa 1988). Therefore, trauma or ischemia induced alterations in ion concentration may regulate the functional state of a major dendritic cytoskeletal protein and in turn modulate neuron morphology and function.

Experimental observations suggest that the dendritic cytoskeleton may be altered in traumatic brain injury and ischemia. For example, 3 hours after moderate fluid percussion injury, quantitative Western blot evaluation of MAP2 levels in rat hippocampal tissue, demonstrates a significant decrease in MAP2 levels (Taft et al 1992). By contrast, histological studies evaluating the pathophysiology of traumatic brain injury, demonstrate that the function and viability of axons and the axonal cytoskeleton is often damaged before neuron somal or dendritic change is observed (Povlishock et al 1992). These conflicting reports, however, suggest that axons and dendrites may be differently damaged or vulnerable following injury or ischemia.

Degradation of the dendritic cytoskeleton, in experimental models of ischemia, can be an early indicator of neuron damage and correlates immunohistochemically with neurons destined to die. For example, 3 minutes after right common carotid artery occlusion in the gerbil, ischemic

changes can be detected in dendrites of the subiculum CA1 region of the ipsilateral hippocampus using immunohistochemical labels for MAP2. Detection of ischemic changes using hematoxylin and eosin stains occurs in a more delayed fashion (Kitigawa et al 1989).

Dendrites and neurodegenerative conditions.

Alzheimer's disease is the most prevalent cause of dementia in industrialized countries and a leading cause of premature death or institutionalization in people greater than 65 years old (Evans et al 1989, Perry 1992). Symptomatic Alzheimer's disease is characterized histopathologically by neuron loss, aberrant somatodendritic sprouting (Ihara 1988), neurofibrillary tangles (NFT) and extracellular deposits of β -amyloid protein (β -AP). Quantitative studies suggest that these lesions correlate with the degree of dementia (Blessed et al 1968).

Substantial evidence suggests that dendritic pathology may be implicated in Alzheimer's disease. 1) In the aging brain an increase in dendrite growth is observed in areas of neuron loss; this age related increase, however, is not observed in Alzheimer's disease (Buell and Coleman 1976, Coleman and Flood 1986, Hanks and Flood 1991). 2) In neuritic plaques a cytoskeletal protein, tau, that is normally found in axons, is dislocated into the somatodendritic compartment where it colocalizes with MAP2 (McKee et al 1989). 3) Senile plaques from patients with Alzheimer's disease demonstrate immunohistochemical labeling for heparan sulfate and chondroitin sulfate proteoglycans (Snow and Wright 1989, DeWitt et al 1993); these proteoglycans do not support dendrite growth *in vitro* (Lafont et al 1992). 4) The addition of

β -amyloid protein to rat hippocampal neurons in culture results in dystrophic dendrite formation (Pike et al 1992).

Deposits of β -amyloid protein present in senile plaques of patients with Alzheimer's disease (Spillantini et al 1990, Quon et al 1991, Perry 1992) may contribute to dendrite injury. For example, β -AP has been demonstrated to enhance glutamate and calcium ionophore induced toxicity of cortical and hippocampal neurons in culture, apparently by potentiating the influx of calcium and destabilizing intracellular calcium metabolism (Mattson et al 1992). Neurons in the mammalian CNS use calcium as a second messenger that mediates a variety of adaptive responses and morphologic plasticity, including dendrite growth. Inability to maintain intracellular calcium within critically normal levels results in dendrite retraction (Mattson et al 1989, Mattson 1992).

Alterations in dendrite structure are associated with many other neurodegenerative conditions such as malnutrition (Jacobsen 1991), intrauterine growth retardation (Rees and Harding 1988), developmental deprivation and lead exposure (Petit et al 1983), and human mental retardation (Purpura 1975). Furthermore, excitatory amino acids, which as discussed above may render dendrites particularly vulnerable to pathologic insult, have been suspected as mediators of neuronal injury in many chronic neurodegenerative conditions including Huntington's disease, AIDS dementia complex, and amyotrophic lateral sclerosis (Lipton and Rosenberg 1994). Thus, it is of critical significance to understand dendrite development, not only to answer fundamental questions in developmental neurobiology, neurophysiology, memory and learning, but also to devise novel strategies

for intervention and rational treatment for acute neurologic disorders and chronic neurodegenerative conditions.

DENDRITE DEVELOPMENT

Introduction.

The process by which a morphologically simple neuroblast develops into a morphologically complex, mature neuron involves several stages. First, neuroectoderm containing a uniform population of neuroepithelial precursors is formed by induction of undifferentiated ectoderm by mesoderm. The neuroepithelial precursor cells then diversify into immature neurons and glial cells, and following mitosis, immature neurons migrate away from the ventricular germinal zone. After reaching their final position, neurons extend axons and a primitive dendritic arbor. When the axon encounters an appropriate target, further dendrite outgrowth occurs and complex but precise synaptic contacts are formed. Finally, interaction with the environment results in functional refinement and modification of neuron morphology. The sequence of events is well described; however, the signals that initiate and control the events are not well understood (Purves and Lichtman 1985, Jacobsen 1991, McConnell 1991 and 1992).

Decisions that control neuron phenotype may occur at any of the developmental stages described above (Levitt et al 1981, McConnell 1991, Schlagger and O'Leary 1991, McConnell 1992, Goodman and Shatz 1993). The final outcome of a cell's phenotype, however, appears to be determined by a complex interplay between cell lineage, including the inherited pattern of gene regulation and cytoplasmic factors that control gene regulation, and environmental factors including, positional information, cell-cell interactions, cell-cell signaling and diffusible factors (Raff 1989, McConnell 1991).

The cerebral cortex is that part of the brain that has expanded most during primate evolution; when do cortical neurons elaborate their dendritic arbor? Golgi, ^3H thymidine autoradiographic, and ultrastructural studies demonstrate that cortical neurons first elaborate dendrites following completion of migration. The leading process of a bipolar migrating neuron becomes the apical dendrite, other dendrites develop *de novo*. Primary dendrites arise directly from the cell body late in embryogenesis. Subsequently, during the early postnatal period, secondary and tertiary branches appear, and finally dendritic spines develop (Ramon Cajal 1954, Shoumakis and Hinds 1978, Miller 1988). These morphologic observations are consistent with immunohistochemical and protein biochemical analysis that demonstrate that MAP2, a dendrite specific cytoskeletal protein, is first detected once neurons have reached their final position in the developing mouse cortex (Crandall et al 1986).

While much is known about axon development, relatively little is known about the factors that regulate dendrite growth. Growth of dendrites could come about in one of several ways. First, the emergence of dendrites from the cell body and the pattern of branching may occur as a matter of chance. Second, the specificity of dendrite growth may be determined by factors intrinsic to the cell including its genetic endowment or cytoskeleton. Third, dendrite growth may be regulated by interactions between the neuron and its environment including the local cellular environment, extracellular matrix, cell adhesion molecules, neurotrophic factors, and neuronal activity. Finally, dendrite growth may represent an interplay between factors intrinsic and

factors extrinsic to the cell. This chapter will review potential mechanisms of dendrite growth.

Dendritic ontogeny recapitulates phylogeny.

The form of the dendritic tree becomes more elaborate during evolution. Ramon y Cajal (1894) first suggested this concept at the end of the last century; recent morphologic observations of neurons in the superior cervical ganglion (Purves et al 1986, Voyvodic 1987, Purves et al 1988) support his hypothesis. First, in the rat, the size and complexity of the dendritic arbor is directly proportional to the animal's body size. Second, comparative studies of the mammalian superior cervical ganglion in several species demonstrate that there are differences in species related to differences in body size. Dendritic arborizations increase in length and complexity as the animal grows; an increase in body size, however, does not correlate with an increase in the number of autonomic neurons (Purves et al 1988).

Genes and dendrite growth.

Direct control of dendrite development by specific genes has not been demonstrated, however, evidence is accumulating that suggests genes or gene products may directly or indirectly influence dendrite growth. For example, when pAntp, a 60 amino acid long polypeptide that corresponds to the homeodomain of *Antennapedia*, a *Drosophila* homeotic gene, is added to embryonic rat mesencephalic neurons (Joliot et al 1991) or chick spinal cord motoneurons (Bloch-Gallego et al 1993) in culture, enhanced neuron differentiation is observed. The neuronotrophic activity of pAntp is linked to the propensity of the polypeptide to bind to cognate sequences in homeoprotein regulated genes (Le Roux et al 1993). Indirectly this may

influence dendrite growth as northern blot analysis and transfection experiments demonstrate that the expression of amyloid precursor protein, which can regulate axon growth (Sola et al 1993, Moya et al 1994), can be downregulated in cultured cells expressing the mouse homeoprotein Hox 3.1 (Violette et al 1992).

Intrinsic control of dendrite growth: arguments for and against.

Several observations, including fractal analysis have been taken to imply that the point of origin of axons and dendrites and their initial outgrowth are determined by factors intrinsic to the neuron (Schade et al 1962, Van der Loos 1965, Wise et al 1979, Bartlett and Banker 1984a, Banker and Waxman 1988, Steward 1989a, Jacobsen 1991, Neale et al 1993): 1) dendritic maturation occurs in a predictable temporal and spatial pattern, 2) improperly aligned neurons conserve their dendritic morphology, and 3) neurons deprived of their normal axonal input form morphologically distinct axons and dendrites.

Morphologic observations suggest that dendritic maturation proceeds in a ventrodorsal or inside out sequence: 1) dendrites of neurons in the ventral lateral geniculate nucleus mature before those of the dorsal lateral geniculate nucleus, 2) cortical neuron dendrites mature later than the dendrites of neurons located in central nuclei that project to the cortex, and 3) dendrites of neurons located in deeper cortical layers mature before those located in superficial layers (Schade et al 1962, Wise et al 1979, Jacobsen 1991). While these observations may imply that factors intrinsic to the cell determine the pattern of dendrite growth, transplant studies using neurons that are not fully differentiated, indicate that the position of the neuron can define its phenotype (Renfranz et al 1991, Schlagger and O'Leary 1991, McConnel 1992).

For example, when an immortalized cell line from the hippocampus that expresses the intermediate filament nestin, typically found in proliferating cells, is transplanted into neonatal hippocampus or cerebellum, the morphologic characteristics acquired by the transplanted cells are characteristic of neurons and glia found at the implantation site (Renfranz et al 1991).

Neurons typically give rise to one axon and a predictable number of dendrites that appear to originate at predictable locations. For example, the main dendritic trunk of cortical pyramidal neurons, originates from the apex of the pyramidal shaped cell body. By contrast, the axon and basal dendrites emerge from the basal pole. Most pyramidal cells are oriented so that apical dendrite points to the cortical surface and the axon toward the white matter. Sometimes pyramidal cells are misaligned and rotated 180 degrees; however, the cell still extends identifiable apical and basal dendrites (Van der Loos 1965). This observation implies intrinsic control of dendrite elaboration, however, it is not clear to what extent the local cellular environment influences process outgrowth.

Neurons deprived of their normal targets, afferent input, or contact with other neurons and glia continue to elaborate dendrites. For example, dissociated hippocampal neurons in culture develop in defined stages characterized by the formation of neurites and differentiation into morphologically distinct axons and dendrites (Bartlett and Banker 1984a, Dotti et al 1988). Considerable dendrite growth is observed and quantitative assessment of the dendritic branching observed *in vitro*, indicates that it does not differ significantly from either predicted branching or dendritic branching

observed *in vivo* (Banker and Waxman 1988). These experiments implied that the establishment of neuronal polarity was intrinsic to the neuron and corresponded to the expression of a neuronal maturation program. The neurons, however, were grown in the presence of medium conditioned by astrocytes which subsequent experiments have found can influence neuron polarity (Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Rousselet et al 1988, Rousselet et al 1990, De Konnick et al 1993).

Further observations of developing neurons in culture suggest that dendrite growth is not determined by intrinsic factors alone. First, morphologic and ultrastructural analysis of developing hippocampal neurons indicate that the axonal or dendritic nature of a newly growing process is not determined at its initiation but only occurs after 3 or 4 days *in vitro* (Cacerces et al 1986, Dotti et al 1988, Deitch and Banker 1993). Second, when a growing axon is transected shortly after differentiation, other processes, including the developing dendrites can develop axonal characteristics (Goslin and Banker 1990, Goslin et al 1990). Finally, a prominent differentiating feature of axons and dendrites is compartmentation of mRNA into dendrites only (Deitch and Banker 1993, Kleinman et al 1994). *In situ* hybridization studies of developing hippocampal neurons, however, demonstrates that newly synthesized mRNA is translocated into both axons and dendrites early in development, but only into dendrites after polarity emerges (Kleinman et al 1994).

Dendrite growth and the cytoskeleton.

In fully differentiated neurons, immunohistochemical studies demonstrate that cytoskeletal constituents are located in either a dendritic or an axonal domain (Binder et al 1984, Cácerces et al 1984b, DeCamilli et al 1984, Huber

and Matus 1984, Shaw et al 1985, Foster et al 1987). For example, MAP₂ is highly enriched in dendrites, whereas neurofilament and tau are concentrated in axons; it was therefore hypothesized that the synthesis and expression of specific cytoskeletal elements is a crucial regulatory factor in process elaboration and differentiation (Cáceres et al 1984a, Matus et al 1986, Matus 1988).

Expression of specific cytoskeletal proteins, however, may not determine process differentiation since the strict compartmentalization of cytoskeletal proteins is observed after the initial elaboration of neurites. For example, quantitative, double-labeled immunohistochemical analysis of developing embryonic hippocampal or cortical neurons *in vitro*, indicates that MAP₂ is found in all processes at initiation of outgrowth; is then coexpressed with neurofilament or tau, and is only selectively expressed in dendrites after about 4 days *in vitro* (Cáceres et al 1986, Kosik and Finch 1987, Pennypacker et al 1991). Furthermore, the expression of MAP₂ is not sufficient to induce the formation of dendrites. For example, in culture medium containing serum, sympathetic neurons develop a dendritic arbor that expresses MAP₂. In serum free medium the neurons are usually unipolar and elaborate an axon; MAP₂ expression, however, is still observed (Higgins et al 1988).

Neurons may become polarized following selective stabilization of microtubules (Kirschner and Mitchison 1986, Rousselet et al 1990). According to this hypothesis, microtubules of unpolarized cells are unstable; an external signal impinging on one region of the cell causes a local biochemical change that alters microtubule stability or density in that region and subsequently results in elongation. Whether microtubules determine, or are only features

of dendrite differentiation is not clear. For example, ultrastructural analysis of developing hippocampal neurons indicates that differences in microtubule polarity orientation and density first appear after axons and dendrites have developed other differentiating features such as sorting of ribosomes (Deitch and Banker 1993).

A role for the cytoskeleton in process differentiation and subsequent dendrite growth suggests that the somatic cytoskeleton carries a template for neuron polarity. Since axonal microtubules are all oriented in the same direction, the position of the microtubule organizing center (MOTC), might determine polarity. In cultured hippocampal cells the position of the MOTC correlates with an indentation in the nuclear membrane; during morphologic differentiation, however, the position of the MOTC does not correlate with the position of the axonal growth cone (Dotti and Banker 1992).

Dendrite growth: the role of afferents, synapses and neurotransmitters.

Dendrites often develop in parallel with the ingrowth of afferents and formation of synaptic contacts. For example, in the developing dentate gyrus of the rat, differentiation of granule cell dendrites occurs during the same period as ingrowth of afferents and synaptogenesis (Bloom 1972, Cowan et al 1980). Furthermore, the number of afferents may be an important factor that determines the size and complexity of the dendritic tree (Perry and Linden 1982, Leventhal et al 1989, Sanes et al 1992a). For example, if a region of retinal ganglion cells in the primate retina is surgically depleted, enlargement of the remaining ganglion cells and dendrites is observed in the cell poor region suggesting that the dendrites are competing for the available afferents (Leventhal et al 1989).

The role of afferents on dendrite growth and maintenance is well documented. For example, partial removal of excitatory afferents to the nucleus laminaris of the chick brainstem auditory nucleus results in rapid and selective atrophy of deafferented dendrites with little or no effect on other dendrites (Rubel et al 1981, Deitch and Rubel 1984). In the autonomic nervous system, dendritic retraction is observed after axotomy, however, following reinnervation the dendritic arbor re-expands (Yawo 1987).

Synapses are regarded as the unique characteristic of neurons for information storage and transfer; they may also provide developmental signals. The synaptotropic theory posits that dendritic growth and branching are initiated and maintained by the synapses formed on growth cones of dendrites (Vaughan 1989). Synapse formation may primarily modulate dendrite branching. For example, in the absence of synaptic activity, dendrites of cultured cerebellar Purkinje cells continue to elongate. Once synaptic contact, identified by electrical activity, is made, dendrite elongation ceases and branching begins. When endogenous electrical activity is blocked by the administration of tetrodotoxin dendrites continue to elongate (Schilling et al 1991). These tissue culture observations are consistent with *in situ* ultrastructural observations that suggest that synaptic contact is particularly important for the growth of tertiary dendrites and dendritic spines (Harris and Stevens 1988).

How do afferents or synapses modulate dendrite growth? The axon terminal, once it reaches its target may provide a retrograde signal that initiates or maintains dendrite growth (Yawo 1987). For example, retrograde transport of

nerve growth factor (NGF) is observed in the autonomic nervous system (Levi-Montalcini 1987). When NGF is administered to sympathetic neurons *in vivo*, or vertebrate sensory neurons *in vitro*, an increase in dendritic arborization is observed (Snider 1988, De Koninck et al 1993). Alternatively, neurotransmitters may provide cell extrinsic signals to neurite outgrowth in the vertebrate CNS (Mattson et al 1988a, 1988b, and 1989). For example, in isolated hippocampal neurons *in vitro*, glutamate can cause selective regression of dendrites through a calcium dependent mechanism without significantly affecting either axon growth or neuron survival (Mattson et al 1988a, 1989b, and 1989). Calcium may be an important second messenger in neuron modulation of dendrite growth. In addition to mediating the effect of glutamate on dendrite elongation, intracellular calcium homeostasis is altered at the time of synapse formation in cultured Purkinje cells, when, as noted above, dendrite elongation ceases and branching begins (Schilling et al 1991).

Can dendrite growth occur without afferent or synaptic influence?

Several observations suggest that the parallel development of dendrites and afferents is not invariable. First, neuronal form can develop in the absence of neuronal activity. Tetrodotoxin which blocks neuron activity is released by the *Taricha* newt; when pieces of the *Taricha* embryo are grafted to embryos of Ambystomid urodeles, the host embryos are paralyzed until the toxin wears off. Despite the absence of neural activity, morphologically normal spinal cord motoneurons are formed (Twitty and Johnson 1934). Second, *in situ* observations using intracellular injections of horseradish peroxidase, lipid soluble tracers, or quantitative Golgi analysis demonstrate that dendritic projections, although immature are established before the arrival of afferents

in a variety of developing systems including the sympathetic ganglia (Voyvodic 1987), spinal cord (Snider et al 1992), dorsal lateral geniculate nucleus (Sutton and Brunso-Bechtold 1993), brainstem (Hammer et al 1981), and cortex (Wise et al 1979, Petit et al 1988).

Tissue culture studies provide further evidence that some aspects of dendrite growth can occur without afferents. For example, isolated hippocampal neurons that develop *in vitro* without afferents can form dendritic arbors similar to that seen *in vivo*. (Barlett and Banker 1984a, Banker and Waxman 1988). In addition, dendrite elongation may be diminished by afferents *in vitro*. For example, decreased dendrite growth is observed from hippocampal neurons when they are grown on a bed of their afferents, axons from the enterorhinal cortex (Mattson et al 1988). Dendritic branching, however, may require afferent or synaptic activity. For example, when the density of cultured hippocampal neurons is increased, or cultured cerebellar neurons establish synaptic contacts (Schilling et al 1991), dendritic branching, but not length, is enhanced.

Neuron activity determines the final form of the dendritic arbor.

The observations described above suggest that afferent or synaptic activity is not always required for the initial development of dendrites, but may be necessary for the full development of a mature dendritic arbor. There is substantial evidence that the final form of the dendritic arbor depends on the presence and function of afferents (Smith 1981, Greenough et al 1985, Black et al 1990, Kolb and Gibb 1991, Sanes et al 1990 and 1992a, Jones and Schallert 1994). For example, in the weaver mouse, a mutation that affects differentiation of cerebellar granule cells, Purkinje cells fail to receive their

normal innervation; dendrites are much shorter and less branched than those observed extending from normal Purkinje cells (Rakic and Sidman 1973).

Afferent function depends, in large part, on the environment; the environment an animal encounters, such as sensorimotor deprivation or increased functional activity, can lead to alterations in size, structure, or orientation of dendrites (Globus et al 1973, Greenough et al 1985, Purves et al 1986, Black et al 1990, Jacobsen 1991). For example, when rats are raised in an enriched environment, a greater density of dendritic spines is observed on pyramidal neurons in the visual cortex than when the rats are raised in a deprived environment (Globus et al 1973). Visual deprivation results in the redistribution of visual cortex stellate cell dendrites. Normally these dendrites arborize extensively in the fourth cortical layer where the majority of lateral geniculate body afferents terminate. By contrast, after unilateral enucleation, stellate cell dendrites avoid the fourth layer and arborize in other layers of the visual cortex (Valverde 1968).

How does neuron function influence dendritic architecture? Observations in several systems demonstrate that during the course of development and maturation, refinement, rather than development, of the dendritic arbor relies on the presence of afferents (Smith 1981, Greenough et al 1985, Black et al 1990, Kolb and Gibb 1991, Sanes et al 1990, 1992a and 1992b, Jones and Schallert 1994). Hypertrophic responses, such as increased dendritic arborization and complexity, primarily at higher order branches (Greenough et al 1985, Coleman and Flood 1986, Leventhal et al 1989, Black et al 1990, Sanes et al 1992a, Jones and Schallert 1994) and increased spine density

(Globus et al 1973, Harris and Stevens 1988) or regressive events, such as a decrease in the number of primary dendrites, (Smith 1981), total process length (Weiss and Pysh 1978), branch points, (Rogowski and Feng 1982, Sanes and Siverls 1991), or number of dendritic spines (Ramo et al 1988) have been described in a wide variety of systems and may account for functional refinement of the dendritic arbor. For example, the dendritic arborization of the lateral superior olive (central auditory nucleus) in the gerbil varies along its tonotopic axis, being more constrained at higher sound frequency projection regions (Sanes et al 1990). Electrophysiologic and Golgi impregnated quantitative morphologic observations demonstrate that these differences occur after the response to airborne sound, and are primarily manifest by a reduction in total dendritic length and number of branch points (Sanes et al 1992b). The number of primary dendrites, however, does not change significantly, consistent with the concept that at least a primitive dendritic arbor, possibly subject to intrinsic or other environmental signals, can develop before neuronal activity.

Glial cells influence dendrite growth.

The local cellular environment can mediate neuron form (Renfranz et al 1991, Schlagger and O'Leary 1991, Grove et al 1992, McConnel 1992, Reh 1992, Marvin and McKay 1993). For example, in the retina, progenitor cells differentiate according to the cells that surround them rather than an intrinsic mechanism (Reh 1992).

Neurons are surrounded by glial cells; in the vertebrate CNS glial cells are between 10 and 50 times more numerous than neurons (Pope 1978, Nauta and Freitag 1986). Interactions between neurons and glia occupy a central role

in the function of the nervous system (Barres 1991, Martin 1992, Finkbeiner 1993) and are of great importance in determining the structural organization and development of the brain. For example, during development glial cells, particularly astrocytes, have been demonstrated to be important for: neuron survival (Banker 1980), neuron migration (Levitt and Rakic 1980, Rakic 1988, Gasser and Hatten 1990), establishment of brain cytoarchitecture (Snow et al 1990a and 1990b, Steindler 1993), neurite outgrowth (Noble et al 1984, Fallon 1985, Tomaselli et al 1988), axon guidance (Silver et al 1982, Silver and Ogawa 1983, Norris and Kalil 1991), establishment of neuron polarity (Denis-Donini et al 1984), and synaptogenesis (Rouget et al 1993).

Do glial cells have a role in dendrite growth? Experimental observations in the peripheral and autonomic nervous systems suggest that both the presence or absence of glia can influence dendrite morphology. For example, when cultured in the absence of glia, rat sympathetic neurons do not develop dendrites, although they extend axons. When glial cells are added the sympathetic neurons promptly elongate their dendritic arbor (Bruckenstein and Higgins 1988, Tropea et al 1988, Johnson et al 1989). By contrast, the presence of glial cells inhibits the extension of dendrites from neonatal rat sensory neurons in culture. Dendrite growth is observed when the glial cells are removed (De Konnick et al 1993).

In the CNS, tissue culture experiments suggest that dendrite morphology can be modified by the glial environment. For example, embryonic mouse mesencephalic neurons when cocultured with astroglia develop different morphologic, immunohistochemical, and ultrastructural dendritic characteristics depending on the anatomic origin of the astroglia. When

grown on, or in medium conditioned by homotypic mesencephalic astroglia, mesencephalic neurons grow both an axon and an extensive dendritic arbor. By contrast, when mesencephalic neurons are grown on, or in medium conditioned by heterotypic striatal astroglia, axon growth occurs but poor dendritic arborization is observed (Denis-Donini et al 1984, Chamak et al 1987, Auttilo-Touati et al 1988, Rousselet et al 1988 and 1990).

Apart from the experiments described above, the role of glial modulation of dendrite growth from neurons has not been extensively investigated. Although ample evidence exists that astroglia can influence dendrite morphogenesis, the mechanism of this influence remains unclear. Do astroglia support cortical neuron dendrogenesis? Do astroglia derived from various regions of the CNS support dendrite growth or are there regional differences in astroglial support of dendrite growth? Do astroglia initiate, maintain or refine the dendritic arbor? Does astroglial age influence dendrite growth? Do reactive astroglia, that are characteristic of the injured brain, influence dendrite growth? Do astroglia mediate dendrite growth by cell-specific interactions or by the release of diffusible factors? The experiments described in this study will attempt to answer some of these questions using tissue culture, quantitative immunohistochemical techniques, and protein biochemistry.

The role of nutrition, hormones and opioids on dendrite growth.

Whereas the predominant influence on dendrite growth is derived from the surrounding cellular environment, neuronal activity and factors intrinsic to the neuron, several other factors have been observed to influence the shape of the dendritic arbor. Among others, these factors include nutrition,

glucocorticoid, thyroid, and sex hormone levels, endogenous opioids and retinoic acid (Lauder 1983, Fischer et al 1986, Hauser et al 1989, Goldstein et al 1990, McEwan 1991, Jacobsen 1991). For example, the most striking feature of malnutrition on the cerebral or cerebellar cortex of rats is a reduction of dendritic spine density (Jacobsen 1991). Along similar lines, the total number of dendritic spines present on cerebellar neurons is decreased in fetal sheep suffering intrauterine growth retardation, in part, because the length, but not the number of granule cell dendrites and the branching density and branch length of Purkinje cell dendrites is reduced (Rees and Harding 1988).

The nervous system regulates and responds to the endocrine system; in turn hormones can influence dendrite growth (Goldstein et al 1990, McEwan 1991). For example, dendrite morphology is influenced by levels of sex hormones in both the male and the female. In the male, dendrites of the spinal bulbocavernosus nucleus motoneurons extend and retract in response to testosterone levels throughout the rat's life (Goldstein et al 1990). In the female rat, dendritic spines present on neurons in the ventromedial hypothalamus and the hippocampus appear and disappear during the course of the ovarian estrous cycle in response to levels of estradiol. By contrast, estradiol does not induce significant changes in dendritic spine density in the male (McEwan 1991).

Factors such as nutrition, hormones and opioids appear to refine the dendritic arbor rather than primarily promote dendrite growth (Rees and Harding 1988, Hauser et al 1989, Jacobsen 1991, McEwan 1991). For example, in neonatal rats, continuous blockade of endogenous opioid receptors by the exogenous antagonist, naltrexone, significantly increases dendritic spine density, but not

primary dendrite number elaborated by cerebral cortical neurons (Hauser et al 1989). Other factors, such as retinoic acid, may play an important role in the induction of dendrite growth. For example, retinoic acid induces the extension of branched and unbranched neurites in neuroblastoma cells (Fischer et al 1986). Furthermore, retinoic acid is among the few factors known to modulate homeogene expression (Boncinelle et al 1991). Since homeogenes specify positional information in the developing and adult mammalian spinal cord and brain (Awgulewitsch and Jacobs 1990, Simeone et al 1992 Prochiantz et al 1993), it is conceivable that an interrelationship between genetic factors such as homeogenes and extrinsic factors such as retinoic acid may account for the anatomical specificity observed in mesencephalic or striatal glial promotion of mesencephalic neuron dendrite growth.

Towards a theory of dendrite development.

The variety of factors, described in the preceding sections, that can influence dendrite development emphasize that there is probably no general theory of dendrite morphogenesis, except at the most abstract level. Dendrite development may occur in three stages: 1) initiation of dendrite outgrowth, 2) establishment and maintenance of basic structure, the primary dendritic arbor, and 3) establishment of the final dendritic arbor, including secondary and tertiary branches and its subsequent refinement and remodeling. Each stage in dendrite development may be influenced by different factors: 1) intrinsic factors may initiate growth, 2) the local cellular environment may influence the primary structure, and 3) neuron activity and the environment, including such diverse factors as nutrition and the level of stimulation, may determine the final dendritic structure. The principle hypothesis of the

experiments reported in this study is that the local astroglial environment influences the primary dendritic arbor extended by a neuron. Ultimately, however, the cumulative effect of different factors, that work in concert on the same cell, orchestrate the final structure of each neuron's dendritic arbor.

METHODOLOGY

Glial cells.

The abundance of cellular phenotypes, and the anatomical, physiologic, and biochemical complexity of the mammalian CNS presents a barrier to elucidating the function of individual cells, the interactions that occur between cells, and the development of cellular morphology. Glial cells *in vivo* are particularly difficult to identify and study since many of the approaches used to study neurons such as electrophysiology, selective lesioning, or retrograde labeling can not be readily applied (Levison and McCarthy 1991).

Many methods exist to isolate individual cells; primary tissue culture provides a means of studying large volumes of pure cells in which variables affecting the cell can be simplified, controlled and manipulated and a means by which growth and inhibitory factors of interest can be isolated (Levison and McCarthy 1991). The technique of cell culture was developed at the beginning of this century (Harrison 1910). During the last 15 years, cell culture has been used extensively to study glial function, particularly function of the most numerous type of glial cell, astrocytes (McCarthy and DeVellis 1980, Levison and McCarthy 1991). The ability to study glia in culture has resulted, in part, from refinements of *in vitro* techniques and the development of immunological markers that unambiguously identify glial cells and subtypes (Raff et al 1978, Eng 1985). Much of our knowledge of the functional properties of various glial cells are derived from tissue culture studies (Hansson 1988, Lilien and Raff 1990, Barres 1991, Hatten et al 1991,

Kapfhammer and Schwab 1992, Kimelberg 1992, Martin 1992, Eddlestone and Mucke 1993, Finkbeiner 1993, Murphy et al 1993, McMillan et al 1994).

Several lines of evidence suggest that glia *in vitro* and *in vivo* may be similar: 1) glia in culture express many of the same ultrastructural properties observed *in vivo* (Kimelberg 1983, Bovolenta et al 1987, Peters et al 1991), 2) the most numerous type of glial cell, astrocytes, express similar quantities of the astrocyte enriched protein, glial fibrillary acidic protein (GFAP, Eng 1985), and the astrocyte specific enzyme, glutamine synthetase (Schousbee 1981), *in vitro* and *in vivo*, 3) electrophysiologic properties are similar *in vitro* and *in vivo* (Kimelberg 1983, Hansson 1988), 4) the timing of glial differentiation (Lillien and Raff 1990) and maturation appears similar *in vivo* and *in vitro*, provided serum is present in the culture media (Hatten 1984, Williams et al 1985, Smith et al 1986, Smith et al 1990), and 5) astrocytes in culture release a large variety of neuropeptides, neurotransmitters, cytokines and growth factors; although this *in vitro* observation may represent an artifact, injured or reactive astrocytes *in vivo* have also been observed to release many of these same factors (McMillan et al 1994).

Glial cells are divided into two major classes by size and embryology: microglia and macroglia. Microglia are small cells that originate from the mesoderm and generally are mobilized after injury to the nervous system. Macroglia are larger cells derived from the neural plate and consist of three principle types: 1) astrocytes, that can be specifically identified by labeling with antibodies against GFAP (Dahl and Bignami 1976, Eng 1985), 2) the myelin producing cells of the CNS, oligodendrocytes, that can be identified by antibodies against galactocerebroside (Raff et al 1978), and 3) Schwann cells,

the principle glial cell of the peripheral nervous system. Astrocytes are the most numerous glial cell and occupy 50% of the brain's volume (Pope 1978, Nauta and Freitag 1986, Schwarz and Marini 1992). The culture techniques used in the experiments described in this study are designed to obtain purified type 1 astrocyte cultures (McCarthy and de Vellis 1980, Raff et al 1983, Levison and McCarthy 1991), or in the case of the retina, a specialized form of astrocyte, the Müller glial cell (Sathy 1985, Cameron and Rakic 1991).

Astrocytes can be readily identified at the light microscopic level. In addition astrocytes contain an unique intermediate filament, GFAP, that permits unambiguous immunohistochemical identification of astrocytes (Dahl and Bignami 1976, Eng 1985, Cleveland and Hoffman 1991). Several classes of astrocytes, that differ in appearance and location can be found in the mature brain. Broadly, astrocytes can be divided into protoplasmic astrocytes, found in the gray matter, or fibrous astrocytes that are more abundant in white matter (Peters et al 1991).

Tissue culture studies suggest an alternate view of broad astrocyte (or astroglial) subtypes based on cell lineage (Raff et al 1983, Miller et al 1989, Raff 1989). In the lineage model astrocytes are designated as type 1 or type 2 based on immunologic and morphologic criteria. These subtypes were first described in the optic nerve (Raff et al 1983) and subsequently have been observed in other regions of the nervous system, including the cerebral cortex, cerebellum and spinal cord (Miller and Raff 1984, Williams et al 1985, Behar et al 1988, Ingraham and McCarthy 1989, Schwarz and Wilson 1992). Type 1 astrocytes are found in gray matter and are proposed to be the cell culture equivalent of protoplasmic astrocytes, whereas type 2 astrocytes are

regarded as the cell culture equivalent of fibrous or perinodal astrocytes (Miller and Raff 1984, French-Constant et al 1986). Whether the type 1 or type 2 astrocyte lineage applies to all CNS regions (Hirano and Goldman 1988, Goldman and Vaysee 1991, Fok-Seang and Miller 1992, Black et al 1993), or is relevant *in situ* (Skoff and Knapp 1991) is not clear, therefore the terms are best reserved for astroglia in tissue culture only.

In general, the properties displayed by astroglia in culture have been verified *in vivo* (Schwarz and Marini 1992); there are, however, several important caveats to consider when interpreting results obtained from tissue culture experiments. First, astroglia in culture are generally prepared from immature brain. Although the origin and properties of cultured astroglia obtained from adult and neonatal rat brain appear similar, it is unclear whether cultured astroglia should be considered mature or immature cells (Norton et al 1988, Norton and Farooq 1989). Second, astroglia in culture are removed from their normal environment and so can not interact with other neural cells. Depending on whether they are cultured alone, or with other neural cells, in particular neurons, astroglia exhibit different physiologic properties (Corvalan et al 1990, Barres et al 1990a and 1990b) and morphologies (Hatten 1985, Mason et al 1988, Ingraham and McCarthy 1989, Gasser and Hatten 1990). The relevance of neuron induced astroglial morphologic changes is not known (Hatten et al 1991). Third, astroglia in culture are exposed to serum, whereas *in vivo*, the blood brain barrier shields astroglia from blood derived factors; the presence of serum can influence astroglial differentiation (Morrison and de Vellis 1981, Raff et al 1983, Ingraham and McCarthy 1989, Raff 1989, Landis et al 1990). Fourth, astroglia in culture are in an artificial environment; astroglial proliferation, differentiation, morphology and

antigenicity may all be affected by the composition of the culture media and substrate (Dahl 1981, Hatten et al 1991).

Astrocytes occupy a central role in the formation, maintenance and function of the nervous system (summarized in Table 1). To examine the influence of astroglia on dendrite growth from CNS neurons several experimental techniques, including: 1) primary tissue culture and chimeric coculture in which purified mouse cortical neurons were cultured with rat astroglial monolayers or medium conditioned by rat astroglia, 2) image intensification and quantitative computer assisted immunohistochemical and morphologic analysis, and 3) protein biochemistry were used. The experimental techniques are described in detail in Materials and Methods.

Neurons.

Mouse neurons can be specifically identified by the immunolabel M6. The M6 antigen is a 35kDa glycoprotein that can be isolated from deoxycholate solubilized membrane fractions of mouse brain (Lagenaur et al 1992). The antibody to M6 is species specific and labels only mouse neurons and their processes. M6 has been extensively studied in interspecies transplants (Lund et al 1985) and to determine cell fate and commitment of retinal progenitor cells (Reh 1992). Chimeric coculture was therefore used to: 1) minimize neuron influence on dendrite growth, as a small number of readily identifiable neurons could be plated in a high density glial environment, and 2) prevent inadvertent analysis of neurons that may have contaminated the glial cultures. M6 was used in combination with immunolabels specific for proteins found in axons or dendrites. Prior to using the various antibodies, their presence in mouse cortex was confirmed by Western blot analysis and

the sequence of their developmental expression was examined by quantitative immunohistochemical techniques (chapter 8).

**TABLE 1: SUMMARY OF KNOWN OR POTENTIAL FUNCTIONS OF
ASTROCYTES IN THE CENTRAL NERVOUS SYSTEM**

| FUNCTION | REFERENCE |
|--|--|
| DEVELOPMENT | |
| Neuron survival | Banker 1980, O'Malley et al 1992 |
| Regulate development of multipotent progenitor cells | Raff 1989 |
| Neuron migration | Levitt and Rakic 1980, Rakic 1988, Gasser and Hatten 1990 |
| Establishment of brain cytoarchitecture | Snow et al 1990, Steindler 1993 |
| Neurite outgrowth | Noble et al 1984, Fallon 1985 |
| Axon guidance | Silver et al 1982, Norris and Kalil 1991 |
| Establishment of neuron polarity | Denis-Donini et al 1984 |
| Modulate neuron morphology | Prochiantz et al 1990, Qian et al 1992 |
| Synaptogenesis | Rouget et al 1993 |
| Formation of Nodes of Ranvier | French-Constant et al 1986 |
| Regulate myelination | Boyles et al 1985, Raff 1989, Blakemore and Franklin 1991, Dutly and Schwab 1991 |
| MAINTENANCE AND FUNCTION | |
| Form glial limitans | Peters et al 1991 |
| Induce blood brain barrier formation | Janzer and Raff 1987, Joo 1987 |
| Regulate cerebral blood supply | Paulson and Newman 1987, Aoki et al 1991 |
| Regulate vessel caliber | Murphy et al 1990 |

| | |
|--|--|
| Regulate extracellular and interstitial sodium, calcium and chloride concentration | Kimelberg 1983, Barres 1991, Sontheimer et al 1991, Finkheimer 1993 |
| Extracellular potassium buffer | Kuffler et al 1966, Orkand 1977, Barres et al 1990a, Barres 1991 |
| Modulate neuronal ion channels or currents | Black et al 1989, Sontheimer et al 1991, Black et al 1993, Wu and Barish 1994 |
| Acid base balance | Kimelberg 1983, Chesler 1990 |
| Nutrient or metabolite supply for neurons | Lowry et al 1964, Tsacopoulos et al 1988 and 1994 |
| Source of neurotrophic factors | |
| - Ciliary neurotrophic factor | Lillien et 1988 |
| - Epidermal growth factor | Gómez-Pinilla et al 1988 |
| - Fibroblast growth factor | Ferrara et al 1988 Gonzalez et al 1990, Woodward et al 1992 |
| - Glial maturation factor | Lim 1985 |
| - Insulin like growth factor | Rotwein et al 1988 |
| - Interleukins | Beneviste et al 1990, Wesselingh et al 1990 |
| - Nerve growth factor | Furukawa et al 1986, Lu et al 1991, Saad et al 1991, Yoshida and Gage 1991, Zafra et al 1992 |
| - Platelet derived growth factor | Mapstone 1991, Yeh et al 1991 |
| - Transforming growth factor | Fallon et al 1990, Wesselingh et al 1990, Saad et al 1991 |
| Source of plasma proteins | Kalderon et al 1990, Zahs et al 1993 |
| Source of proteases and protease inhibitors | Kalderon et al 1990, Deschepper et al 1991, Zahs et al 1993 |
| Source of neuropeptides | Shinoda et al 1989, Intebi et al 1990, Zahs et al 1993 |

| | |
|--|---|
| Enkephalin metabolism | Shinoda et al 1989 |
| Nitric oxide/endothelium derived relaxing factor metabolism | Murphy et al 1990, 1993 |
| Prostanoid metabolism | Pearce et al 1989 |
| Iron metabolism | Orita et al 1990, Zahs et al 1993 |
| Glutamate and ammonia metabolism | Norenberg 1979, Schousbee 1981, Kaneko et al 1988 |
| Modify synaptic transmission and plasticity | Pomeroy and Purves 1988, Theodosis and Poulain 1989, Smithson et al 1990 |
| Release neurotransmitters, neuromodulators and neuroactive substances | Barres 1991, Flott and Seifert 1991, Gallo et al 1981, Martin 1992 |
| Uptake of excitatory amino acids | Murphy and Pearce 1987, Ahmed et al 1990, Ortega et al 1991, Clark and Mobbs 1992, Schwarz 1993, Holzwarth et al 1994 |
| Uptake of neurotransmitters and neuromodulators including GABA, taurine, norepinephrine, dopamine, serotonin, acetyl-choline, histamine, adenosine, prostoglandins, benzodiazepines, vasoactive intestinal peptide, glucagon, and somatostatin | Hansson 1988, Lerea and McCarthy 1989, Bevan 1990 |
| Neuron-glial and glial-neuron signaling | Cornell-Bell et al 1990, Barres 1991, Ortega et al 1991, Dani et al 1992, Van den Pol et al 1992 |

INJURY AND REPAIR

| | |
|-----------------------------------|--|
| Wall off necrotic tissue | Berry et al 1983, Mathewson and Berry 1985, Reier et al 1992 |
| Phagocytosis | Noske et al 1982 |
| Control deposition of scar tissue | Logan et al 1992 |

| | |
|--|---|
| Neuroprotection | Vilbulsreth et al 1987, Sugiyama et al 1989, Mattson and Rychlik 1990, Rosenberg 1991, Rosenberg et al 1992, Giulian et al 1993 |
| Provide lipids for membrane biosynthesis | Diedrich et al 1991 |
| Immune response | Hertz et al 1990 |
| Diminish excess iron loads | Orita et al 1990, Zahs et al 1993 |
| Neurite growth | David et al 1990, Kawaja and Gage 1991 |
| Inhibit axon growth | Berry et al 1983, Reier et al 1983, Liuzzi and Lasek 1987 |
| Growth factor production | Nieto-Sampedro et al 1982, Manthorpe et al 1983 |
| - Epidermal growth factor | Nieto-Sampedro et al 1988 |
| - Fibroblast growth factor | Finklestein et al 1988, Gomez-Pinilla et al 1992, Logan et al 1992 |
| - Insulin like growth factor 1 | Garcia Estrada et al 1992 |
| - Nerve growth factor | Bakhit et al 1991 |

Table 1: The table lists published data or reviews describing known or potential functions, or evidence for a function for astrocytes in the CNS. Astrocytes were thought to be important in brain function at the beginning of the 20th century (Lugaro et al 1907), but, until recently, were relegated to a static, passive support function. Today, there is substantial experimental evidence that astrocytes are dynamic partners in CNS function and actively participate in the formation, maintenance, and repair of the central nervous system. The following broad categories of astrocyte function are recognized: 1) development of neuron and CNS architecture, 2) exchange mechanism between capillaries and neurons, 3) extracellular ionic micro-environment regulation, 4) provision of neuronal nutrition or trophic support and regulation of neuronal metabolism, 5) synaptic activity regulation, 6) intercellular signaling, and 7) repair and protection of the injured CNS.

MATERIALS AND METHODS

TISSUE CULTURE

Dissociated cerebral cortex neuron cultures.

Highly enriched populations of dissociated cerebral cortical neurons were prepared from the brains of mouse embryos. Pregnant Balb/c mice (E18) were killed by decapitation following CO₂ anesthesia and the embryos removed under sterile conditions. Frontal cerebral cortex was dissected in sterile Hank's balanced salt solution (HBSS). After carefully removing the meninges, the cortex was minced into 1mm pieces and dissociated into a single cell suspension using the following protocol: 1) pieces of frontal cortex were placed in 5 ml of Ca²⁺, Mg²⁺ free HBSS; 2) the pieces of cortex were incubated at room temperature in this solution for 10 minutes and then 0.5 ml of a 2.5% trypsin solution was added; 3) the incubation was continued for another 10 minutes on a shaking device at 37°C. At the end of incubation, the trypsin was inactivated by adding 0.5 ml of fetal bovine serum; 4) the cells were then concentrated by centrifugation (1500rpm, 5 minutes) and the supernatant was removed; 5) fresh medium (2 ml) was added and the pellet was triturated to a single cell suspension; 6) the cells were plated in DMEM:F12 (without glutamate or aspartate, GIBCO, Grand Island, NY) supplemented with insulin, (25µg/ml); transferrin, (100µg/ml); putrescine, (60uM); selenium, (30nM); progesterone (20nM) and penicillin/streptomycin. For all experiments, the cortical neurons were plated at low density (1×10⁴cells/ml) and maintained for 5 days either on astroglial substrates (see glial cell cultures) in serum free medium (above), or poly-D-lysine (50-100 µg/ml) coated coverslips in the presence of continuously conditioned

astroglial medium (see noncontact transfilter experiments) or astroglial conditioned medium (see conditioned medium). Cells were grown at 37°C in an atmosphere of 5% CO₂

Glial cell cultures.

Astroglial cultures from different CNS regions, were obtained from postnatal day 4 Sprague Dawley rat pups. This particular age was chosen to facilitate dissection of discrete anatomical regions and maximize astroglial purity (McCarthy and De Vellis 1980, Kimelberg 1983, Hatten 1985, Giulian et al 1988, Misson et al 1991, Schwartz and Wilson 1992). After careful removal of the meninges and as much white matter as possible, pieces of frontal cerebral cortex, mesencephalon, olfactory bulb, striatum, spinal cord or the entire retina were dissociated on a shaking device in Ca²⁺ Mg²⁺ free HBSS containing 0.25% trypsin at 37°C for 20 minutes. At the end of the incubation the trypsin was inactivated with 0.5 ml of fetal bovine serum. The cells were collected by centrifugation (1500rpm, 5 minutes) and the supernatant removed. Fresh medium (2 ml) was added and the cells triturated to a single cell suspension. The cells were then plated (approximately 1x10⁵ cells/ml) in 25 ml plastic tissue culture flasks and maintained in 10% serum containing medium (DMEM & Hams F-12, GIBCO) until they reached confluence (10-14 days). Glial cultures were purified for astroglia or Müller glia using a modification of published methods (Levinson & McCarthy 1991). Briefly, confluent monolayers were shaken overnight (250rpm for 18 hours at 37°C) to remove loosely adherent phase bright cells. The remaining attached cells were first washed in HBSS and then exposed to 0.25% trypsin in Ca²⁺ Mg²⁺ free HBSS for 30 seconds. The cells were then incubated in Ca²⁺ Mg²⁺ free HBSS at 37°C for 5 minutes and further detached cells discarded. The

remaining adherent cells were removed from the flasks following incubation in Ca^{2+} Mg^{2+} free HBSS containing 0.1% trypsin at 37°C for 5 minutes. After this incubation, the trypsin was inactivated with fetal bovine serum and the remaining cells were rinsed in serum containing medium and dispersed. The cells were then plated (approximately 1×10^5 cells/ml) onto glass coverslips, coated with either poly-D-Lysine/laminin (20 $\mu\text{g}/\text{ml}$) for Müller glia or poly-D-Lysine for other glial subpopulations, placed in individual wells of a 24 well plate, and allowed to form a confluent monolayer (2-5 days). Once a confluent monolayer was formed mouse cortical neurons were introduced. Meningeal fibroblast monolayers were derived from the meninges of P4 rat pup meninges and prepared as described above. For all the co-culture experiments, the cells were maintained in chemically defined medium as described above for dissociated neuronal cultures.

Mature and reactive glial cell cultures.

Mature astroglia, derived from cortex and mesencephalon, were obtained from P12 rat pups (Smith et al 1986 and 1990) as described in glial cell cultures above and allowed to mature in culture for 2 weeks before use. Reactive astroglia were obtained from P4 or P12 rat pups that had received a penetrating cortical lesion, under anesthesia on P1 or P9 respectively. Rats were anesthetized by hypothermia and CO_2 narcosis. After removing the meninges, a series of stab wounds to a depth of 3-5mm were made with a flame heated 27 gauge needle in the left frontal cortex. Wounds were approximately 5mm long by 3mm wide. Bleeding was stopped with gelfoam (Upjohn) and the incision was closed. After rewarming, animals were returned to nursing mothers. Non lesioned animals were used as controls as several studies have demonstrated reactive glial changes, of varying severity,

in regions of the brain distal to a lesion, including in the contralateral hemisphere (Steward et al 1990, Moumdjian et al 1991, Garcia Estrada et al 1992, Anezaki et al 1992). Cells obtained from lesioned animals were purified as described in glial cell cultures above.

Non contact transfilter experiments.

To test for the effect of secreted factors from astroglia, purified astroglial cells were obtained as described for glial cultures above. Cells were then plated at high density (approximately 1×10^5 cells/ml) on Millicell culture inserts (0.4 μ m) in 24 well plates and allowed to grow to confluence. For non contact coculture experiments, cortical neurons were plated at low density (1×10^4 cells/ml) onto poly-D-lysine (50-100 μ g/ml) coated coverslips located beneath the millipore filter. Small legs elevate the filter over each coverslip thus neurons are continuously exposed to astroglial conditioned medium. Cells were grown at 37°C in an atmosphere of 5% CO₂.

Conditioned medium.

Astroglial conditioned medium was obtained from purified, confluent mesencephalic or cortical glial monolayers. The monolayers were first washed extensively with HBSS and serum free medium and then incubated for 48 hours in serum free, chemically defined medium (see neuron cultures). The collected conditioned medium was concentrated 5-fold by Speedvac ultracentrifugation (Savant Instruments, Hicksville, NY), pooled and after being sterile filtered (0.2 μ m, Millipore, Bedford MA), was reconstituted in the same volume of chemically defined medium prepared for neuronal cultures. Conditioned medium was stored at -70°C and thawed before use.

PROTEIN BIOCHEMISTRY

SDS-PAGE and immunoblotting of mouse cortex.

Brain homogenates were prepared from E18 cortex in 50mM Tris, pH 8.0, 50mM NaCl, 1% NP-40, 100µg/µl phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO) and 1µg/µl aprotinin. Samples were solubilized in 3% SDS, 10% 2-mercaptoethanol, 65mM Tris-HCl, (pH 6.8) and boiled for 2 minutes. After boiling, 15µl of sample was loaded onto a 8% SDS-polyacrylamide gel and electrophoresed according to the Laemmli procedure using a minigel system (Biorad, Richmond CA). The gel was then electroblotted onto nitrocellulose using a sandwich transfer apparatus (Biorad) in 25mM Tris and 0.25% SDS. Blots were processed to block nonspecific protein binding in 5% dry milk, PBS (pH 7.5) and 0.1% Tween followed by incubation overnight with the primary antibody in 5% dry milk, PBS (pH 7.5) and 0.1% Tween at 4°C (anti-MAP2 1:100, anti-NF-H 1:200, anti-M6 1:10) Blots were washed three times and incubated in secondary antibodies at room temperature. After subsequent washing the blot was developed using the ECL system (Amersham Life Sciences, Little Chalfont, England).

Denaturation of conditioned medium.

Medium conditioned by cortical astroglia was heat inactivated at 90°C for 15 minutes. Heated medium was centrifuged at 3000g for 10 minutes and supplemented with a mixture of hormones and proteins (see neuron medium). Conditioned medium was also treated with trypsin (0.1%; GIBCO) at 37°C for 30 minutes. The reaction was stopped by adding 0.1% fetal calf serum. The medium was supplemented with a mixture of hormones (see neuron medium).

Protein concentration.

Medium conditioned by cortical astroglia was concentrated by spinning in Centricon-100 tubes (molecular weight cut off 100kDa; Amicon, Danvers MA) in a refrigerated centrifuge (1000 rpm, 60 minutes; Sorvall, DuPont Instruments, Wilmington DE). Low molecular components of the conditioned medium were removed by dialysis in tubing with a molecular cutoff of 10kDa (Spectrasphore, Spectrum, Los Angeles CA) for 24 hours against sterile 0.5M NaCl at 4°C and then against sterile 0.15M NaCl for 36 hours at 4°C. The dialysate was partially concentrated on polyethylene glycol, desalted on a Sephadex gel filtration column (Pierce, Rockford IL), further concentrated in a refrigerated Speedvac apparatus (Savant Instruments), sterile filtered, and reconstituted in chemically defined medium (see neuron cultures). Protein concentration was estimated by uv spectrophotometry (Beckman Instruments, Fullerton CA.).

Heparin affinity chromatography.

Medium conditioned by cortical astroglia was diluted with PBS (0.01M KH_2PO_4 , 0.15 M NaCl, pH7.4) and applied at a linear flow rate of 15cm/hr onto small heparin-agarose columns (5ml; Biorad). The columns were then washed with application buffer (0.01M KH_2PO_4 , 0.15M NaCl, pH7.4) and coupling determined by measuring the absorbance of the elute and application buffer at 280nm. The bound protein was eluted with 1.5MNaCl in application buffer. The column elutes were desalted on a Sephadex gel filtration column (Desalting Column HR-10/10; Pierce), concentrated in a refrigerated Speedvac apparatus (Savant Instruments), sterile filtered and

reconstituted in chemically defined medium. Total protein was determined by the Pierce Commassie Protein Assay (Pierce).

Protein radiolabeling.

Purified, confluent monolayers were incubated for 48 hours in chemically defined medium. The medium was removed and the monolayer washed with methionine depleted medium. After adding methionine depleted chemically defined medium (MEM, Select-Amine, GIBCO) and ^{35}S methionine (50 $\mu\text{Ci/ml}$; 1600Ci/mmol, Amersham), the monolayers were incubated at 37°C for a further 3 hours.

Protein extraction from astroglial monolayers and conditioned medium.

Astroglial cultures grown to confluence and labeled with ^{35}S methionine were removed from the culture flasks by aspirating off the medium, rinsing the cells with ice cold HBSS and scrapping the flask with a rubber policeman. After centrifugation (1500rpm, 10 minutes) the tissue was placed in single detergent lysis buffer (50mM Tris Cl, pH 8.0; 150mM NaCl; 0.02% Sodium Azide; 100 $\mu\text{g/ml}$ PMSF; 1 $\mu\text{g/ml}$ aprotinin; 1% NP-40) added for 15 minutes at 4°C. Following centrifugation (1500rpm, 10 minutes, 4°C), the supernatant was dissolved in SDS sample buffer and boiled for 5 minutes. Conditioned medium was dissolved in SDS sample buffer and boiled for 5 minutes. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (8 and 12% SDS-PAGE; Biorad). Equal amounts of protein (25 μg) were loaded for each cell extract. Protein concentrations were determined using the Bicinchoninic acid (BCA) protein assay system using bovine serum albumin as a standard (BCA, Pierce). Gels were fixed in 10%

glacial acetic acid, 20% methanol, dried and exposed to xray film (Kodak XAR-2) for 48 hours.

Peptide growth factors.

The following growth factors were tested: Transforming growth factor (TGF , 10ng/ml; Collaborative Research, Bedford MA), Epidermal growth factor (EGF, 10ng/ml; Collaborative Research), Nerve growth factor (NGF, 2.5S and 7S isoform, 200ng/ml; Collaborative Research), Platelet derived growth factor (PDGF, 10ng/ml; Collaborative Research), acidic Fibroblast growth factor (aFGF, 1ng/ml), and basic Fibroblast growth factor (bFGF, 1ng/ml; R&D Systems, Minneapolis, MN).

IMMUNOHISTOCHEMISTRY AND ANALYSIS

Immunohistochemical identification of cells in culture.

Different glial cell types were distinguished using the following monoclonal or polyclonal antibodies: anti-GFAP (glial fibrillary acidic protein, Boeringer-Mannheim, Indianapolis, IN, 1:100), anti-Vimentin (Boeringer-Mannheim, 1:100), OX-42 (Harlan Bioproducts for Science, Indianapolis IN, 1:100), A2B5 (hybridoma supernatant, American Tissue Type Collection, Rockville, MD, 1:10), Thy 1.1 (OX7 clone ascites, Cedar Lane, Hornby, Ontario), Nerve growth factor receptor (NGFR) specific rat monoclonal antibody (RAT 192 Ig, generously donated by Dr. Mark Bothwell, University of Washington, Seattle), Nestin (RAT 401, generously provided by Dr. Susan Hockfield, Yale University, New Haven), galactocerebroside antiserum (Gal-C, Chemicon, Temecula, CA, 1:50), and a polyclonal antiserum raised in rabbits against cellular retinaldehyde binding protein (CRALBP from Dr. J. Sarri, University

of Washington, Seattle). Supernatant from hybridoma cells was used without dilution, while ascites from these cells was typically diluted 1:10. A minimum of 5 coverslips were labeled with each antibody and labeled cells were counted from a minimum of 5 arbitrarily selected fields to determine the cellular phenotypic composition of the glial monolayers.

Mouse neuronal cell bodies and neurites were immunostained with a mouse neuron specific monoclonal antibody, M6 (hybridoma cell line raised in rat and generously donated by Dr. C. Lagenaur, University of Pittsburgh, Pittsburgh, 1:10). Axons were identified with a rabbit polyclonal antiserum to the 200 kDa neurofilament protein (NF-H, Sigma, 1:200). A mouse monoclonal antibody to microtubule associated protein 2, (MAP2, Boeringer-Mannheim, 1:100) was used as a specific marker of dendrites.

Prior to immunostaining, coverslips were fixed in 4% paraformaldehyde (0.1m phosphate buffer, 4% sucrose, pH 7.4, 60 minutes, 20°C) and then washed 3 times in 0.1m phosphate buffer (PBS). In order to visualize cell surface antigens such as those recognized by A2B5, Thy 1.1, M6 or Gal-C, cells were first incubated in 0.1m PBS containing 1% bovine serum albumin (Sigma, 30 minutes, 20°C); to visualize intracellular antigens such as NF-H or MAP2, cells were permeabilized with 0.05% Triton X-100 (0.1m PBS, 1% bovine serum albumin, 30 minutes, 20°C). Primary antibodies were diluted in PBS with or without 0.3% Triton X and incubated for 1 hour at 20°C). After the primary incubation, the cells were washed 3 times in PBS containing 4% goat serum (Sigma). Labeling was detected with fluorescein or rhodamine conjugated antibodies (1:200 in PBS, 1 hour, 20°C). Mouse antibodies were visualized with fluorescein coupled goat anti-mouse Ig (Cappel, Organon

Technika, Durham NC). Rabbit or rat antibodies were visualized using direct or indirect immunofluorescence with rhodamine conjugated goat anti- rat or -rabbit Ig. In some cases indirect immunofluorescent labeling was carried out using biotinylated secondary antibodies (anti-mouse or rabbit IgG, Sigma, 10 µg/ml) followed by avidin-TRITC (Sigma) or streptavidin-FITC (Molecular Probes, Eugene, OR). Omission of the primary antibody eliminated the observed immunoreactivity on the various cultures. Immunostained cultures were washed in PBS and mounted onto glass slides in aqueous mounting solution (Fluomount).

Primary antibodies raised in different hosts were used to perform double-labeling experiments, according to standard published techniques (Beltz and Burd 1989), to quantify neurite growth. Working dilutions of the respective primary antibodies were applied in sequential incubations. Secondary antibodies were not mixed but were presented sequentially to the appropriate primary antibody. Each secondary antibody was labeled with a different fluorochrome: FITC (green), RITC (red) or Texas Red (orange red). Double-labeling was prepared according to the following protocol: 1) first primary antibody, 2) secondary antibody or secondary biotinylated antibody if appropriate, 3) avidin or streptavidin fluorochrome, 4) second primary antibody, and then repeat steps 2 and 3.

Analysis of cortical neuron survival and process outgrowth.

Neuron survival was assessed by estimating the number of viable purified neurons present at 1, 3 and 5 days *in vitro* using both morphologic and immunocytochemical techniques. Six random microscopic fields on five coverslips were examined in three separate cultures of each cellular subtype.

At each time point the number of viable neurons was counted microscopically and expressed as a percentage of the number of viable cells that were initially plated. The initial number of surviving cells was calculated at 3 hours by counting the number of attached cells after the plating medium had been replaced with fresh medium to remove any unattached cells. The number of attached cells 3 hours after plating was taken to be 100%. The results of preliminary experiments demonstrated that viability counts according to these criteria agreed within 5% with counts of cell viability determined with Trypan Blue exclusion.

The growth of neurites from cortical neurons identified by M6, NF-H and MAP2 immunoreactivity was analyzed after 5 days *in vitro*, and in some experiments, where indicated, at 18 hours, 1, 2 and 3 days *in vitro*. Double-labeling immunohistochemical techniques using a combination of: 1) MAP2 and M6, 2) MAP2 and NF-H, or 3) M6 and NF-H were used in all experiments to quantify neurite growth and cytoskeletal protein expression. Filters specific for each emission wavelength were used to block out emission from one fluorochrome while examining the other. A computer generated density profile of each immunolabel was created to determine its presence objectively (Image Analyst; Autamax, Billerica, MA). Briefly, in preliminary experiments we calculated the average width of neurites to specify a plot profile (N). Plot profiles were calculated at right angles to each neurite and only pixels within the profile were included in the computation of density. Density readings were converted into calibrated units using standard curves that had been predetermined against a standard, calibrated optical density tablet. Measurements of density were treated as relative values and units were arbitrary. Density was derived from the formula: $N \times \text{mean density} - N \times$

background density (N = number of pixels in selection). Immunoreactive cells were examined in six different microscopic fields selected at random on a minimum of 5 coverslips for each experimental condition. To avoid ambiguity only isolated neurons whose cell bodies were immunoreactive for MAP2, and whose cell bodies and processes were not in contact with other neurons were quantified. At least three experiments for each test condition or cell monolayer were carried out; approximately 100 neurons were examined in each experimental condition.

For measurements of neurite length, neurons were examined at a final image magnification of 400X. Fluorescent images of the neurons were recorded with an image intensifier coupled to a CCD video camera (Motion Analysis, Eugene, OR) and analyzed with a Macintosh II image processing program (Image Analyst). Neurite lengths were measured by tracing the total length of any neurite extending from a neuron cell body. Recorded lengths were calibrated at the same magnification using a microscope slide micrometer. The number of minor neurites or primary dendrites per cell, the length of major neurites or axons, the length of minor neurites or primary dendrites, the total length of minor neurites or primary dendrites, and, in some experiments, the total neurite length per cell were calculated. Analysis of statistical significance of any observed differences between substrates was done using ANOVA, Student's unpaired T-test, or the Mann-Whitney U procedure.

**THE DEVELOPMENTAL TIME COURSE OF PROCESS SPECIFIC
CYTOSKELETAL PROTEIN EXPRESSION IN EMBRYONIC MOUSE
CORTICAL NEURONS *IN VITRO***

INTRODUCTION

Most neurons in the vertebrate CNS are polarized and elaborate two distinct types of processes, dendrites and axons which exhibit distinct morphologic, electrophysiologic, cytoskeletal, and biochemical differences (Sargent 1989, Craig et al 1992). One of the most striking differences between axons and dendrites is cytoskeletal composition. In the mature CNS, biochemical and immunohistochemical studies indicate that cytoskeletal proteins are compartmentalized into either an axons or a dendrites (de Camilli et al 1984, Huber and Matus 1984, Carden et al 1985, Crandall et al 1986, Matus et al 1986. It is postulated that the regionalization of cytoskeletal constituents is largely responsible for the complex and diverse shapes of neurons and thus is of critical significance for neuron function.

The precise mechanism that contributes to the segregation of cytoskeletal proteins is largely unresolved (Craig et al 1992). Primary culture systems, in which individual neurites are visible, have been used principally to examine how the neuron cytoskeleton is sorted into either an axon or a dendrite since individual cells can be labeled with immunohistochemical markers against dendrite or axon specific proteins. For example, neurofilament, particularly the highly phosphorylated 200 kDa isoform (NF-H; Carden et al 1985, Shaw et al 1985, Foster et al 1987, Pennypacker et al 1991) or tau (Kosik and Finch 1987) are the major cytoskeletal components found in axons, whereas microtubule

associated protein type 2 (MAP2) is localized to the cell body and dendrites (Cáceres et al 1986, Kosik and Finch 1987, Higgins et al 1988, Pennypacker et al 1991). The identification of axons or dendrites based on the distribution of cytoskeletal immunolabels *in vitro* corresponds to process identification *in situ* and at the ultrastructural level (Banker and Waxman 1988).

When developing neuronal processes are examined *in vitro*, however, strict compartmentation of cytoskeletal constituents is not observed during the initial elaboration of neurites (Cáceres et al 1986, Kosik and Finch 1987, Higgins et al 1988, Pennypacker et al 1991). For example, in rat hippocampal neurons, MAP2 is initially present in both axons and dendrites (Cáceres et al 1986, Pennypacker et al 1991) and can be coexpressed with either NF-H (Pennypacker et al 1991) or tubulin (Cáceres et al 1986) before process differentiation is complete. During the course of neuron differentiation and process development, immunolabel separation is observed; MAP2 staining becomes more intense in dendrites and is progressively lost from the axon. By contrast, immunolabels for tubulin or NF-H are expressed in the axon and are not observed in dendrites. Identification of an axon or a dendrite based solely on immunohistochemical criteria can only be made after about 3 or 4 days *in vitro*. Similar immunohistochemical observations, using antibodies to MAP2 and tau, have been reported for developing rat embryonic cerebral cortical neurons (Kosik and Finch 1987). These immunohistochemical observations in culture are consistent with morphologic observations, using time lapse microscopy, of developing mouse hippocampal neurons *in vitro* (Dotti et al 1988).

The time course of axon and dendrite development and sorting of process specific cytoskeletal proteins in the developing mouse cortex is not known. Therefore we utilized quantitative morphologic and immunohistochemical analysis to examine neurite formation in primary cultures of embryonic mouse cortical neurons to determine the time course of: 1) morphologic differentiation of axons and dendrites, and 2) MAP2 and NF-H segregation into dendrites and axons respectively. The results are consistent with previous observations, and demonstrate that for embryonic mouse cortical neurons *in vitro* MAP2 and NF-H segregate into dendrites or axons respectively, but not at the initiation of process outgrowth.

MATERIALS AND METHODS

Cortical neurons were prepared from the brains of E18 Balb/c mouse embryos, plated at low density and maintained in serum free medium on rat cortical astroglial monolayers as described in chapter 7. The growth of neurites from cortical neurons identified by M6, NF-H or MAP2 immunoreactivity was analyzed after 18 hours, 1, 3 and 5 days *in vitro*. Three experiments for each time point were carried out. A total of 100 immunoreactive neurons, double-labeled with MAP2, NF-H, and M6, were examined at each time point as described in chapter 7

RESULTS

Cytoskeletal and surface antigen recognition in E18 mouse cortical neurons.

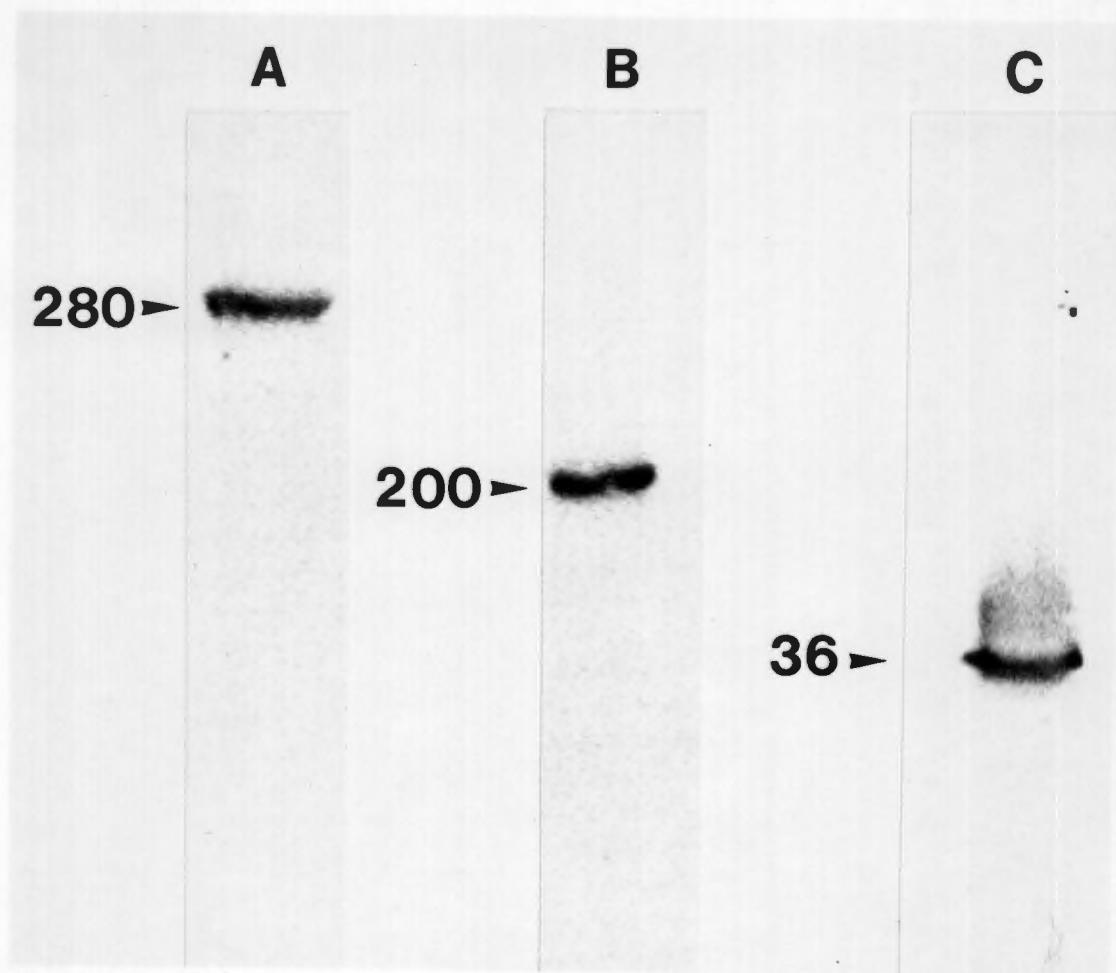
Antibody recognition of neural cytoskeletal constituents and surface markers depends on several factors including: 1) the species from which the tissue was

obtained, 2) the animal's developmental age, 3) the region of the brain examined, and 4) the particular antibody used in the analysis (Shaw and Weber 1982, Sternberger and Sternberger 1983, Pachter and Liem 1984, Dahl and Bignami 1985, Crandall et al 1986, Carden et al 1987, Crandall and Fischer 1989, Pennypacker et al 1991). We therefore examined the presence of MAP2, NF-H and M6 in E18 mouse brain. Cortical extracts were separated on SDS polyacrylamide gels and immunoblotted with the monoclonal antibodies to MAP2, NF-H and M6 intended for immunohistochemical analysis. Figure 1 illustrates the results of this analysis; antibodies against MAP2, NF-H and M6 identified the corresponding protein in brain homogenates derived from E18 mouse cortex.

Neurite identification.

In order to characterize the identity of a neurite as either a dendrite or an axon, light microscopic morphological and immunohistochemical criteria were used. Dendrites elaborated by mature neurons followed a relatively straight course from the cell body and demonstrated immunoreactivity for MAP2 (Cáceres et al 1986, Matus et al 1986, Pennypacker et al 1991). Along its projection, the dendrite tapered; its length being proportional to the diameter of the stem. Branches from dendrites occurred at acute angles. By contrast, mature axons were immunoreactive for the 200 kDa neurofilament protein, NF-H, (Shaw et al 1985, Pennypacker et al 1991) and were more uniform in diameter, longer and less branched and followed a wandering course. Branches when they did occur, were at an obtuse angle.

FIGURE 1: Brain homogenates from E18 mouse cortex were separated on 8% SDS-PAGE gels and electroblotted onto nitrocellulose. The blot was incubated with primary antibodies to a) MAP2, b) NF-H or c) M6 followed by a secondary antibody and demonstrates the expression of each protein marker in E18 mouse cortex.



Morphology of developing axons and dendrites.

A strict division between the immature axon and dendrite, based solely on light microscopic morphology, is not always possible in developing rat cortical or hippocampal neurons *in vitro* (Cáceres et al 1986, Kosik and Finch 1987, Dotti et al 1988, Pennypacker et 1991). The presumptive axon, however, can be differentiated from other processes by its growth characteristics (Dotti et al 1988). Experiments evaluating the response of hippocampal neurons to neurite transection *in vitro* indicate that once the length of one process exceeds the length of others by 10µm or more it is specified as the axon and rapidly acquires axon properties (Goslin and Banker 1990, Goslin et al 1990). Sequential observation and time lapse video microscopy of developing hippocampal neurons demonstrates that this transformation occurs after 18 to 24 hours in culture (Dotti 1988) and is closely correlated with the selective segregation of cellular constituents (Goslin and Banker 1990, Goslin et al 1990, Deitch and Banker 1993, Kleinman et al 1994). Ultrastructural morphologic differences are first visible between 1 and 3 hours after the establishment of polarity (Deitch and Banker 1993). With continued growth the longer process acquires all the morphological, biochemical and immunohistochemical features characteristic of axons and the remaining processes acquire features of dendrites (Dotti et al 1988, Goslin and Banker 1990, Goslin et al 1990, Deitch and Banker 1993, Kleinman et al 1994). Therefore, in early cultures, before immunostaining was segregated, the presumptive axon was defined as that process with the greatest length after the computerized measurement of all neurites of a cell.

To characterize the sequence of morphologic development demonstrated by axons and dendrites elaborated by mouse cortical neurons in culture, we

examined developing E18 neurons and their processes at 18 hours, 1, 2, 3 and 5 days *in vitro* using phase contrast microscopy. Within 18 hours of plating most neurons had extended multiple processes of similar lengths and morphology. By 1 DIV (day *in vitro*) neurons typically extended a single long process and several shorter ones. Subtle morphological differences between axons and dendrites were first observed when the length of the longest neurite exceeded the length of other processes by at least 15 μm . This was first observed at 24 hours *in vitro* on all the various monolayers. Distinct morphologic differentiation of processes was observed in most neurons after 3 DIV and, by 5 DIV, all neurites examined demonstrated either axon or dendrite morphology.

Distribution of cytoskeletal proteins in developing mouse cortical neurons.

Previous studies that have examined the time course of process outgrowth from hippocampal (Cáceres et al 1986, Dotti et al 1986, Pennypacker et 1991) or rat cortical neurons (Kosik and Finch 1987) *in vitro*, indicate that segregation of cytoskeletal elements is not coincident with the elaboration of processes; instead it is achieved after about 3 days *in vitro*. Therefore, to characterize the time course of cytoskeletal protein segregation in mouse cortical neurons and determine the earliest time point at which axons and dendrites from mouse cortical neurons could be distinguished by immunohistochemical criteria, we examined the morphology and segregation of cytoskeletal elements in E-18 mouse cortical neurons, using double-labeling immunohistochemical techniques, at 18 hours and 1, 2, 3 and 5 days *in vitro*. Primary neurites were characterized according to their expression of cytoskeletal proteins, MAP2, NF-H, or both and expressed as a percentage of the total number of neurites examined at each time point. A computer quantified density profile of each

immunolabel was created to determine its presence objectively (Figure 2). To prevent ambiguity on cell phenotype, only cell bodies that expressed MAP2 were included in this quantitative analysis. A total of 100 neurons were examined at each time point. The results are summarized in Table 2.

As described in previous studies (Cáceres et al 1986, Kosik and Finch 1987, Higgins et al 1988, Pennypacker et al 1991), the immunoreactivity for cytoskeletal components is initially not confined to particular neurites. Instead, the immunoreactivity for cytoskeletal components begins to be differentially localized as mouse cortical neurons developed features of axons or dendrites in culture (Table 1 and Figure 3). NF-H expression became more intense in axons and MAP2 labeling more intense in dendrites. The segregation of MAP2 and NF-H to dendrites and axons, respectively, takes place over several days. Although initial signs of segregation are present at 2 DIV, even by 3 DIV, the segregation in detectable immunoreactivity is not complete. For example, 60% of presumptive axons are immunoreactive for both MAP2 and NF-H; MAP2 is present in only the proximal portion of nearly two thirds of these neurites, whereas NF-H labeling is present along the entire length of the presumptive axon, including the growth cone. Thirty seven percent and 3% of presumptive axons are exclusively immunoreactive for NF-H and MAP2 respectively.

By 5 DIV immunohistochemical segregation of these cytoskeletal antigens is complete (Figures 4 and 5). Coexpression of MAP2 and NF-H immunoreactivity, however, still occurs in nearly half the axons. In axons that demonstrate coexpression of immunolabels, MAP2 immunoreactivity is found only in the proximal portion of virtually all processes examined.

FIGURE 2: Fluorescent images of neurons, double-labeled with MAP2 and NF-H, were recorded with an image intensifier coupled to a CCD video camera (Motion Analysis) and analyzed with a Macintosh II image processing program (Image Analyst; Automax). A computer quantified density profile was used to determine objectively the presence of MAP2 or NF-H immunolabeling of neurites elaborated by mouse cortical neurons *in vitro*. The values illustrated are those obtained from a single neuron at 5 DIV. a) - d) demonstrates density of MAP2 immunoreactivity in: a) proximal dendrite, b) distal dendrite, c) distal axon and d) proximal axon. The density of NF-H immunoreactivity is illustrated in: e) axon and f) dendrite.

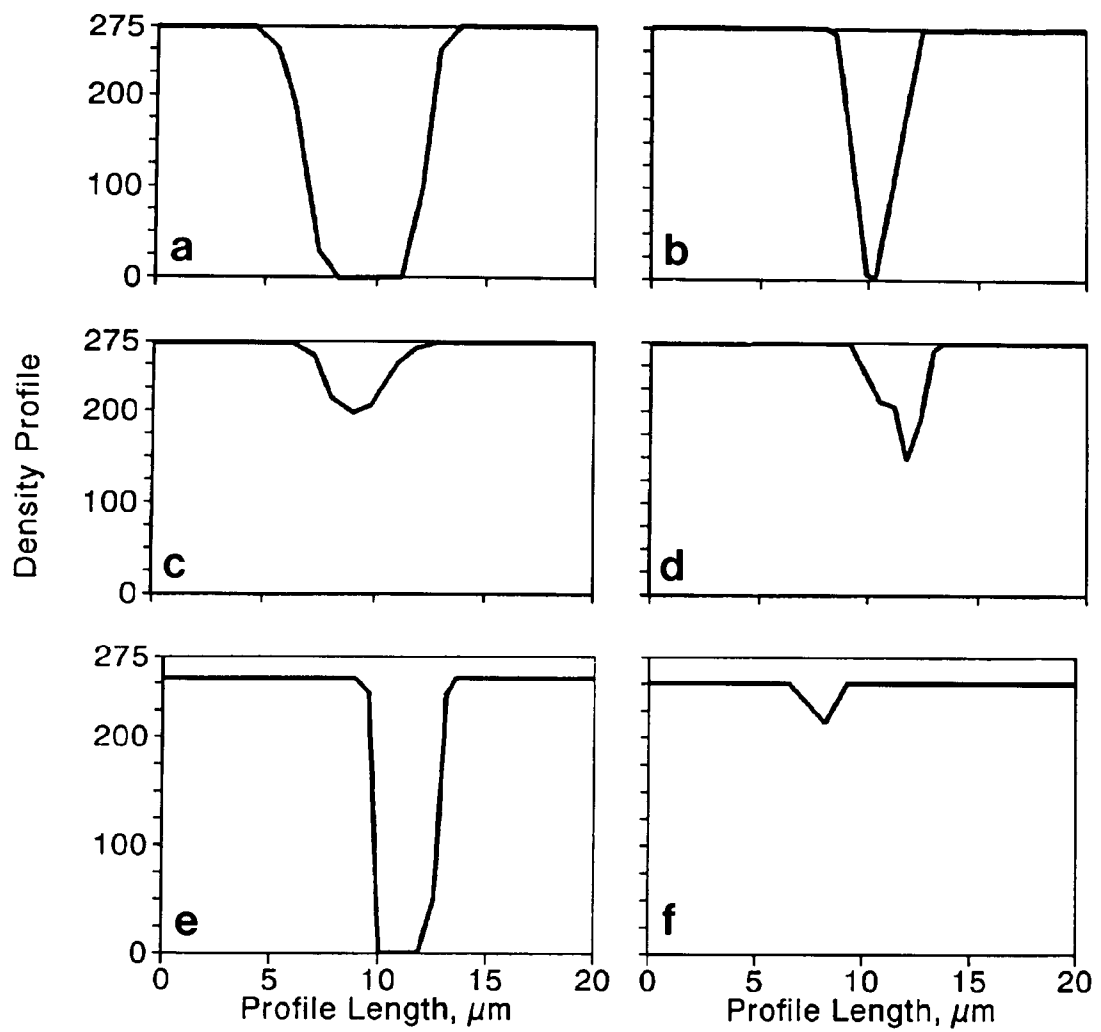
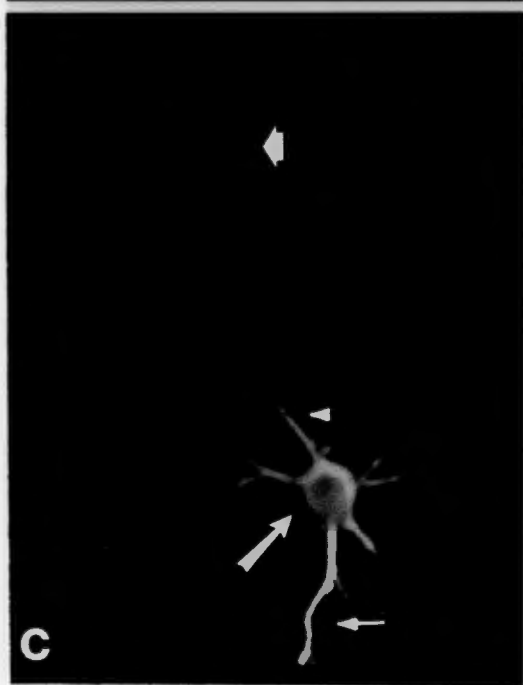
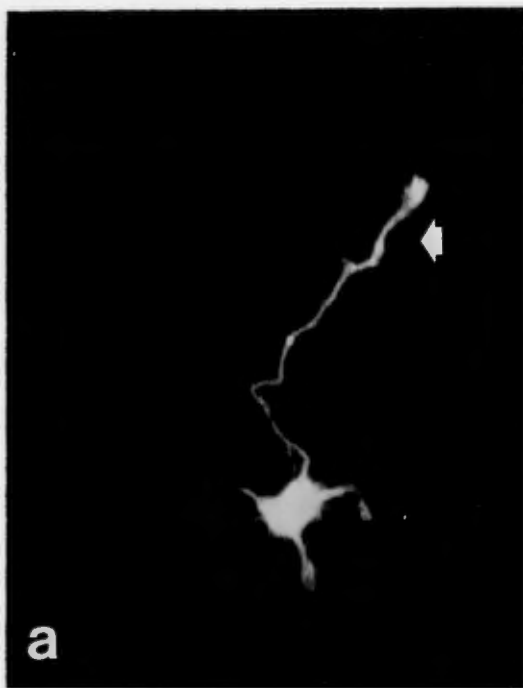


TABLE 2: NEURITE IMMUNOHISTOCHEMICAL CHARACTERISTICS

| | 18 HRS/1 DIV | 2DIV | 3DIV | 5DIV |
|----------------------------|--------------|------|------|------|
| ALL NEURITES | | | | |
| MAP ₂ only | 100 | 58.8 | 54.3 | 65 |
| NF-H only | 0 | 0 | 14.3 | 14.2 |
| BOTH | 0 | 41.2 | 31.4 | 13.3 |
| AXON | | | | |
| MAP ₂ only | 100 | 26.6 | 3 | 0 |
| NF-H only | 0 | 0 | 36.6 | 53.1 |
| BOTH | 0 | 73.3 | 60 | 46.9 |
| MAP ₂ proximal* | 0 | 22.7 | 61 | 80 |
| DENDRITE | | | | |
| MAP ₂ only | 100 | 69.7 | 76 | 98.9 |
| NF-H only | 0 | 0 | 4 | 0 |
| BOTH | 0 | 30.3 | 20 | 1.1 |

Table 2: Segregation of process specific cytoskeletal immunolabels is not coincident with neurite elaboration from mouse cortical neurons in culture, but occurs after at least 3 DIV with progressive loss of MAP₂ expression from axons. Primary neurites that extended from MAP₂ positive neurons were characterized according to their expression of cytoskeletal proteins: MAP₂, NF-H or both and expressed as a percentage of the total number of neurites examined. Presumptive axons and dendrites were characterized according to morphologic criteria or relative length as described in Materials and Methods (n=100 cells from 3 separate experiments each at 18 hrs and 1,2,3,5 DIV; * refers to the percentage of axons expressing both immunolabels in which MAP₂ is only identified proximally). At 18 hrs and 1DIV primary neurites, including the presumptive axon express MAP₂ exclusively. (The results are pooled in one column). By 2DIV there is an increase in NF-H expression but only in neurites immunolabeled by MAP₂. After 3 days in culture, dissociation of immunolabels into respective axon and dendrite compartments emerges with an increase in the number of processes that express NF-H only. Immunohistochemical characterization of neurites is possible by 5DIV. Virtually all dendrites express only MAP₂. Nearly half the axons still express MAP₂; invariably this is exhibited in the proximal axon only. NF-H labeling is demonstrated along the entire axon process.

FIGURE 3: Time course of MAP2 and NF-H distribution in cortical neurons. Corresponding fluorescent photomicrographs of E18 mouse cortical neurons grown for 1 (a and b) or 5 (c and d) days *in vitro* and labeled with MAP2 (a and c) or NF-H (b and d). At 1 DIV MAP2 staining is present in the cell body and all neurites, even in the longest neurite or presumptive axon (arrow). NF-H labeling is not observed (b). Dissociation of immunolabels is present, but not complete at 3 DIV. Morphological characteristics, however, allow identification of the processes. Immunoreactivity has a characteristic distribution at 5 DIV (c and d). MAP2 staining is observed in the cell body (large arrow), straight, short, tapering neurites typical of dendrites (small arrow) and the proximal portion of the axon (small arrowhead). The remaining axon does not contain detectable MAP2 (large arrowhead). There is little NF-H label (d) in the cell body (large arrow) and dendrites (small arrow), but intensive staining in the axon (large arrowhead).



Neurites with the morphologic characteristics of dendrites have a distinct immunologic phenotype; exclusive anti-MAP2 expression occurs in 98.9% of dendrites examined. Although the remaining dendrites coexpress both immunolabels, MAP2 staining is usually far more intense than NF-H expression. Beyond 5 DIV, a mat of axons dominates the culture and prevents accurate analysis of individual neurites along their entire length.

In order to label entire cells and their processes, we used an additional antibody, M6. This antibody recognizes an antigen that is present on the surface of mouse neurons and their processes (Lund et al 1985, Lagenaur et al 1992). Throughout the period examined (between 18 hours and 5 days *in vitro*), M6 immunoreactivity was present in all of the processes. Therefore, at 5 days *in vitro*, a cortical neuronal process was classified as a dendrite if it was immunoreactive for both M6 and MAP2 and/or lacked immunoreactivity for NF-H. Alternatively, at 5 days *in vitro* a cortical neuron process was classified as an axon if it was M6 and NF-H immunoreactive and/or lacked MAP2 immunoreactivity (Figures 4 and 5).

DISCUSSION

In the present experiment we examined developing mouse cortical neurons using immunohistochemical and morphological techniques. The results demonstrate that axons and dendrites elaborated by mouse cortical neurons in culture can be characterized by morphological and immunohistochemical criteria; these characteristics, however, are not present at initiation of process outgrowth but are acquired during development. These observations have several implications: 1) classification of a neurite as either an axon or a

FIGURE 4: Distribution of MAP2, NF-H and M6 in cortical neurons. Corresponding fluorescent photomicrographs of E18 mouse cortical neurons grown for 5 days *in vitro* and labeled with MAP2 (a and c), NF-H (b) and M6 (d). MAP2 staining is present in the cell body (large arrow), straight, short, tapering neurites typical of dendrites (small arrow) and the proximal portion of the axon (small arrowhead). The remaining axon does not contain detectable MAP2 (large arrowhead). There is a paucity of NF-H label (b) in the soma (large arrow) and dendrites (small arrow), but intensive staining in the axon (large arrowhead). M6 labeling (d) is distributed on the cell surface of the soma (large arrow) and all processes and is only found in mouse neurons. (Scale bar = 10 μ m; a and b, c and d).

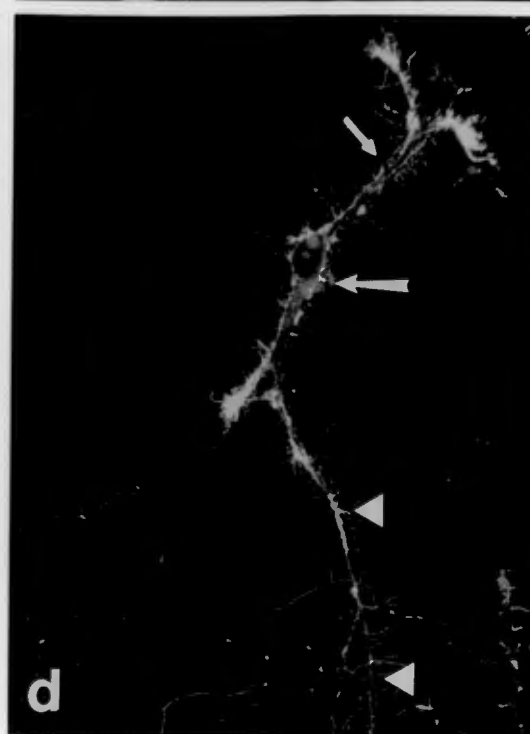
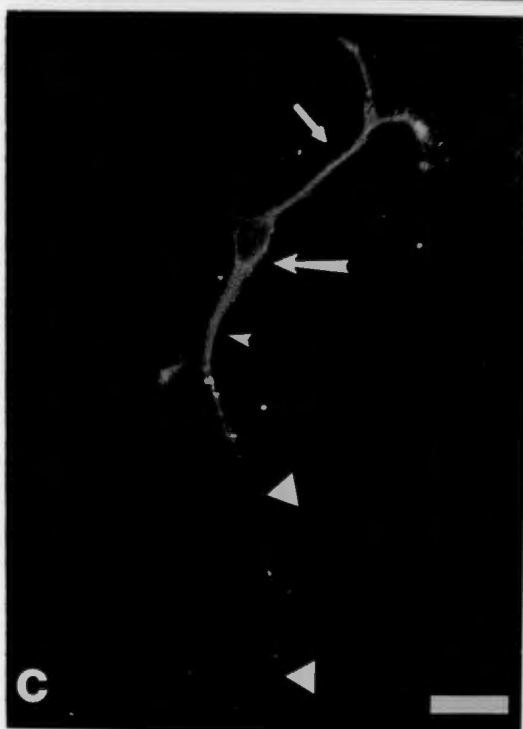
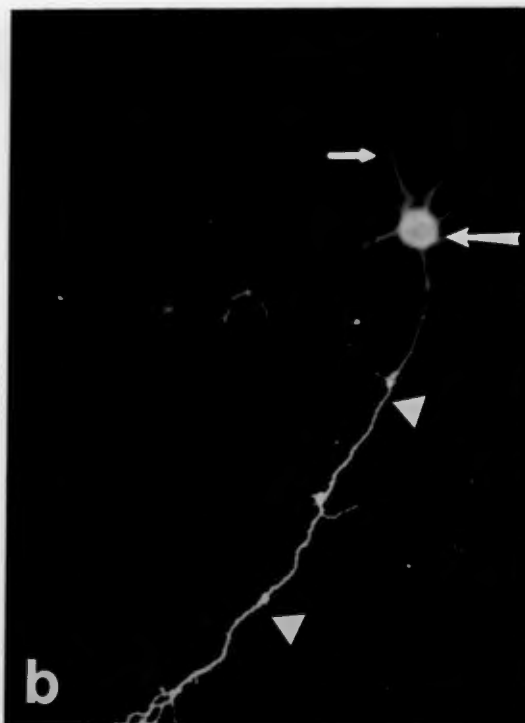
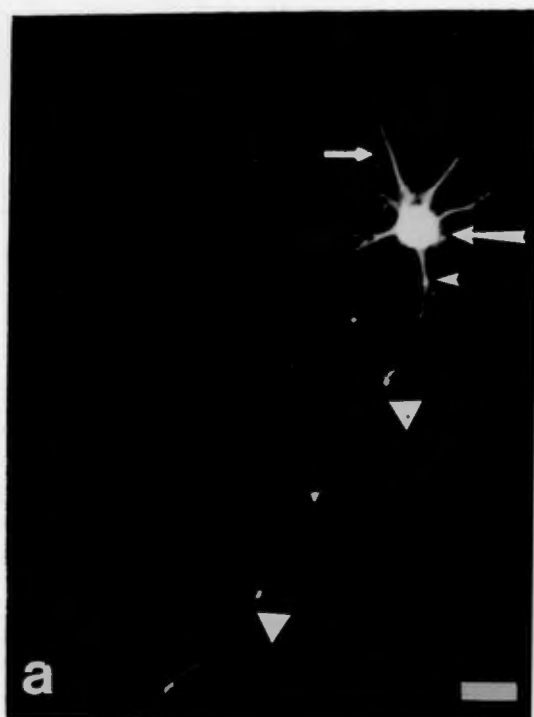
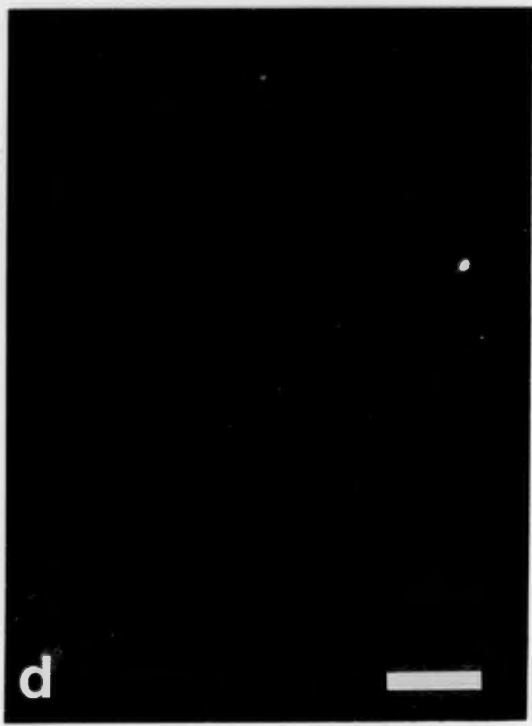
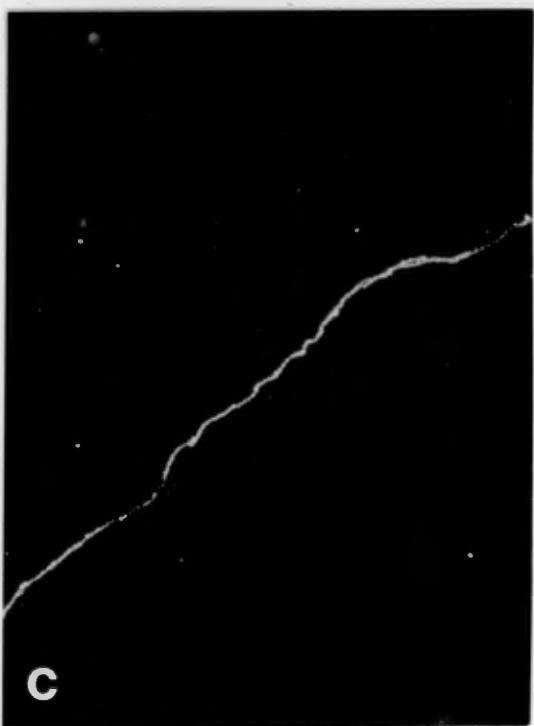


FIGURE 5: Immunohistochemical characteristics of axons. Corresponding fluorescent photomicrographs of axons extended by E18 mouse cortical neurons grown for 5 days *in vitro* and labeled with M6 (a), MAP2 (b and d), and NF-H (c). M6 expression, with patches of continuous labeling that alternate with punctate staining, is detected in the entire axon, including the growth cone (a). NF-H labeling is present as continuous staining along the entire axon (c). MAP2 immunoreactivity is not present in the axon or axonal growth cone (b and d). Occasionally MAP2 immunoreactivity may be detected in the proximal axon. (Scale bar = 10 μ m)



dendrite, using only immunohistochemical criteria, is first possible after 3 to 5 days *in vitro*, 2) nascent neurites are neither axons or dendrites, and 3) cytoskeletal constituents, alone, do not determine the identity of a developing neurite.

MAP2 and NF-H expression in developing mouse cortical neurons.

The expression of MAP2, a dendrite specific marker, is found in all neurites at initiation of outgrowth from mouse cortical neurons *in vitro*. These observations are consistent with immunohistochemical and morphological observations in other systems (Cáceres et al 1986, Kosik and Finch 1987, Higgins et al 1988, Pennypacker et al 1991) indicating that the definite immunohistochemical identity of a neuronal process *in vitro*, is not present at initiation of process outgrowth but is acquired later. For example, MAP2 immunoreactivity has similarly been observed to stain the cell body and all processes uniformly in primary cultures of rat hippocampal (Cáceres et al 1986, Pennypacker et al 1991) or rat cortical neurons (Kosik and Finch 1987) at initiation of process outgrowth. It is unlikely, therefore that the distribution of MAP2, alone, determines which process will become a dendrite. Uniform distribution of MAP2 immunoreactivity in developing neurites, however, is consistent with recent experimental observations which demonstrate that the initial establishment of neurites depends, in part, on MAP2 (Dinsmore and Solomon 1991, Cáceres et al 1992, Brugg et al 1993). For example, expression of MAP2 antisense oligonucleotides in cerebellar neurons suppresses the expression of MAP2 and inhibits the initial outgrowth of neurites (Cáceres et al 1992). By contrast, when antisense oligonucleotides of tau, a cytoskeletal protein found in the mature axon, are added to cerebellar neurons in culture,

elaboration of an axon like process is inhibited whereas exploratory minor neurites continue to extend (Cáceres et al 1991).

Neurofilament, is not detected in developing mouse cortical neurons during the first day *in vitro*. The time when neurofilament immunoreactivity can be detected first in developing neurons, however, is not clearly elucidated. In our culture system we first detected NF-H labeling after 2DIV and only in neurites that expressed MAP2. Coexpression of MAP2 with NF-H (Pennypacker et al 1991) and other axonal markers such as tau (Kosik and Finch 1987) or tubulin (Cáceres et al 1986) has been observed in other systems. Most investigators have reported that NF immunoreactivity is first detected between 1 and 14 after culture (Shaw et al 1985, Foster et al 1987, Pennypacker et al 1991). There are several reasons for these discrepant results: 1) expression may depend on the antibody used (Shaw et al 1985, Dahl et al 1986), 2) the phosphorylation state of NF may determine its detection (Carden et al 1985, Dahl et al 1986, Foster et al 1987) and 3) the presence of glia may influence the expression of NF (Shaw et al 1985). Regardless of the exact timing of neurofilament expression, specific cytoskeletal compartmentation, detectable by immunohistochemical techniques, only occurs after the initiation of process outgrowth with progressive loss of MAP2 immunoreactivity from axons. NF-H expression, on the other hand is separately expressed with greater intensity in axons. At least 48 hours appears to be a critical time point in the selective separation of cytoskeletal immunolabels (Shaw et al 1985, Foster et al 1987, Pennypacker et al 1991). Mouse cortical neurites, *in vitro*, can be classified, by immunohistochemical criteria alone, as either an axon or a dendrite between 3 and 5 days after plating.

Electron microscopic, immunohistochemical and transport studies in the developing optic nerve (Pachter and Leim 1984, Dahl and Bignami 1985) and immunohistochemical and ultrastructural observations in dorsal root ganglia (Shaw et al 1985) indicate that NF-H is not required for the initial outgrowth of neurites or elongation of axons. Instead, NF-H expression may determine axon diameter (Hoffman et al 1985 and 1987, Cleveland and Hoffman 1991). Control of axon diameter is important as it is a critical determinant of conduction velocity. An increase in cross sectional area, therefore is probably necessary only after the process has achieved an adequate length or reached its target and established synaptic stabilization. The axon's diameter then expands and neurofilament expression becomes abundant. NF-H expression is probably not required to enhance process diameter in dendrites as, relative to axons, dendrites grow only short distances. NF-H expression, therefore, is very rarely seen in the developing dendrite.

Synthesis and assembly of cytoskeletal proteins.

After an initial uniform distribution in developing neurites, MAP2, a dendrite specific protein, is selectively lost from the axon. The loss of MAP2 from developing axons could come about in one of two ways: 1) retraction of MAP2 containing axons and subsequent development of a MAP2 free axon, or 2) turnover or redistribution of MAP2 to dendrites. The progressive loss of MAP2 from axons, and gradient of MAP2 expression observed in developed axons in the experiments reported in this study are consistent with redistribution of MAP2, but do not completely exclude the possible retraction of MAP2 containing axons.

The cellular mechanisms that underlie compartmentation of cytoskeletal proteins in neurons is not known (Craig et al 1992, Solomon 1992). Protein synthesis is possible in dendrites (Torres and Steward 1992). Furthermore, the mRNA for MAP2 is present in differentiated dendrites (Kleinman et al 1990, 1994); dendrites may thus be able to locally synthesize and assemble their cytoskeletal components depending on metabolic or structural demand. By contrast, axons are unable to synthesize proteins (Stewart 1989, Torres and Steward 1992); metabolic labeling and immunohistochemical experiments suggest that axon cytoskeletal proteins are synthesized in the cell body (Cleveland and Hoffman 1991, Watson et al 1993).

Where are axonal proteins assembled? Early *in vivo* radiolabeling experiments demonstrated that axonal proteins were assembled in the cell body, however, subsequent experimental techniques suggest that assembly can also occur in the axon growth cone (Bamburg 1986, Cleveland and Hoffman 1991, Tanaka and Kirschner 1991, Solomon 1992). For example, when drugs that inhibit microtubule assembly are locally applied at the growth cone, axon elongation is stopped (Bamburg 1986). The immunohistochemical observations in the experiments described in this report suggest NF-H may be assembled distally near the axonal growth cone. For example, as the axon achieved its specific cytoskeletal identity, the antigen being eliminated, MAP2, receded in a distal to proximal direction toward the cell body. Furthermore, NF-H expression first became abundant distally. Similar immunohistochemical findings have been observed in the developing rat cortex and mouse hippocampus *in vitro* (Kosik and Finch 1987, Pennypacker et al 1991). For example, when hippocampal neurites that subsequently became axons expressed both MAP2 and NF-H during

development, NF-H immunoreactivity was concentrated distally whereas MAP2 was expressed proximally (Pennypacker et al 1991). These immunohistochemical results, however, may also be explained by distal post translational modification, such as phosphorylation, of NF-H.

Extrinsic signals and the cytoskeleton.

The axonal and dendritic cytoskeleton can be modulated, either directly or through activation of second messenger systems that regulate intracellular kinases or calcium, by extrinsic factors including macro- and microglial cells, diffusable factors, the extracellular matrix and cell adhesion molecules (Shaw et al 1985, Chamak et al 1987, Higgins et al 1988, Atashi et al 1992, Damsky and Werb 1992, Thiery and Boyer 1992, Djabali et al 1993, Letourneau et al 1994). Astrocytes may provide important extrinsic signals to the neuronal cytoskeleton. For example, when mesencephalic neurons are cultured in the presence of homotypic astrocytes, the number of MAP2 immunoreactive cells and the intensity of MAP2 expression is increased (Chamak et al 1987). Similarly, NF-H expression in developing hippocampal axons *in vitro* occurs more frequently where the axons are in contact with astroglial cells or in areas where there are large numbers of astrocytes (Shaw et al 1985). These observations provide provocative evidence for a role for glia in the determination of neurite cytoskeletal identity, and perhaps process differentiation, subsequent to initiation of process outgrowth.

How does process differentiation occur? Our results are, in part, consistent with the model adopted by Banker and colleagues (Dotti et al 1988, Goslin et al 1990, Deitch and Banker 1993) and others (Kosik and Finch 1987, Pennypacker et al 1991) and imply that neurons elaborate several immature, non-

committed neurites which subsequently differentiate into axons and dendrites. This model, however, does not fully explain axonal characteristics, such as uniform orientation of microtubules (Baas et al 1987 and 1989, Deitch and Banker 1993) that are observed in all undifferentiated neurites. Neurons, therefore may extend several immature axons, some of which subsequently regress to be followed by dendrite growth, or are transformed into dendrites.

It is possible that some experimental observations described in this report are an artifact of tissue culture as dissociation results in disassembly of axons and dendrites and therefore interferes with the localization of cytoskeletal elements when new processes arise (Matus 1988, Crandall et al 1989, Doering 1992). For example, MAP2 is not observed in axons *in situ* (Matus 1988, Crandall et al 1989), and transplant studies suggest that appropriate interaction between neuron and target is necessary, in some circumstances, to maintain normal cytoskeletal composition, including MAP2 and NF composition (Doering 1992). It is also possible that cytoskeletal proteins may be present but not detectable using immunohistochemical techniques. In addition, since the neurons were obtained from late embryonic brain, it is possible that the cells were already polarized, but as new processes arose were stabilized according to the environment. The observations in this report, however, do have two important implications for the study of process outgrowth: 1) axons and dendrites differ early in the course of development, but not necessarily at initiation of outgrowth and 2) external signals can modulate neuron morphogenesis.

**REGIONAL DIFFERENCES IN ASTROGLIAL DERIVED FACTORS THAT
PROMOTE DENDRITIC OUTGROWTH FROM MOUSE CORTICAL
NEURONS IN VITRO**

INTRODUCTION

A number of factors have been observed to be important in regulating the growth of processes from neurons in CNS. The factors necessary for the generation of a neuron's form derive (1) from constraints intrinsic to the cell and (2) from molecules within the complex microenvironment of the cell. The most well studied examples of extrinsic factors are those derived from glial cells. Descriptive studies and both *in vivo* and *in vitro* manipulations indicate that neuron-glia interactions influence the generation and elaboration of neurites and provide a preferred substrate for neurite extension (Noble et al 1984, Fallon 1985, Tomaselli et al 1986, Neugebauer et al 1988, Tomaselli et al 1988, Smith et al 1990, Norris and Kalil 1991, Baird et al 1992).

In addition to their general support of neurite growth from both central and peripheral neurons, there is increasing evidence that glia can regulate axon and dendrite growth differently. A variety of co-culture experiments (summarized in Table 3) have been performed by several different laboratories, and the results have for the most part been consistent with the hypotheses that homotypic astroglial cells support a greater number of more highly branched dendrites, whereas target derived astroglia promote axon growth (Prochiantz et al 1990, Rousselet et al 1990, Qian et al 1992). For example, Prochiantz and colleagues (Denis-Donini et al 1984, Chamak et al

1987, Autillo-Touati et al 1988, Rousselet et al 1988 and 1990, Prochiantz et al 1990) have found that in the embryonic rodent CNS, both mesencephalic and striatal neurons elaborate a greater number of primary neurites when co-cultured with astroglia derived from the homotypic brain region. In addition to the effects on dendrite morphology, Rousselet et al (1990) observed that mesencephalic neurons extended longer axons in medium conditioned by striatal (target) derived astroglia than by mesencephalon derived astroglia. More recently, Qian et al (1992) has extended these observations by demonstrating that axon growth from either hippocampal or spinal neurons was specifically enhanced by target derived astroglia.

Several co-culture experiments, however, have provided results that are in part inconsistent with this simple model. Whereas homotypic glia can induce dendrite growth from rat sympathetic neurons comparable to that observed *in situ* (Tropea et al 1988), heterotypic, cerebral cortical astroglia can also support extensive neurite growth from these cells (Johnson et al 1989). Furthermore, Denis-Donini and Estenov (1988) reported that dopaminergic neurons of the substantia nigra exhibited more extensive neurite outgrowth on astroglia derived from the olfactory bulb than on homotypic or target derived astroglia. Also, whereas Qian et al (1992) found that axon growth was differentially regulated by astroglia derived from different brain regions, they failed to find any effects on dendrite number, similar to that described by Rousselet et al (1990) for mesencephalic neurons.

Thus, it is unclear whether local or target glia are always better than heterotopic glia at promoting appropriate neuron morphology, or alternatively, the relevant difference between glial cells cultured from

TABLE 3: SUMMARY OF PUBLISHED DATA ANALYZING THE INFLUENCE OF ASTROGLIA FROM DIFFERENT REGIONS OF THE CENTRAL NERVOUS SYSTEM ON NEURITE OUTGROWTH

| AUTHOR | ANALYSIS | ASTROGLIA | NEURON | NEURITE # / L | AXON | DENDRITE # | DENDRITE L | CONCLUSIONS |
|-----------------------------------|--|--|--|--|---|---|--|--|
| Le Roux & Reh 1994, this study | 5 DIV M6 MAP2 NF-200 | Rat P4 SC Mes Str OB Ret Ctx | Mouse E18 Ctx | | 172.7±6.7µ 181.4±6.5µ 170.9±8.0µ 179.2±8.9µ 161.9±4.3µ 184.7±7.4µ | 1.6±0.2 1.4±0.1 1.4±0.1 2.7±0.3 3.5±0.2 4.3±0.3 | 16.7±1.8µ 16.1±1.8µ 12.0±1.4µ 19.3±1.6µ 19.5±1.3µ 16.6±1.1µ | Primary dendrite number, and to a lesser extent length, but not axon growth is influenced by astroglia in a region selective manner. In part, this is mediated by a diffusable factor. |
| | | Contact & non contact. | | | Increased 'axon' length observed on astroglia. No effect observed with conditioned medium. Target neurons stopped neurite growth. | | | In absence of target neurons, astroglia provide + signal to axons. Cell-cell contact required. |
| Baird et al 1992 | 2 DIV M6 (MAP2) (NF-200) | Mouse Cerebellum P3-6 Non contact. | Mouse P0 Pons explant | | | | | |
| | | | | | | | | |
| Qian et al 1992 | 4 DIV MAP2 NF-200 | Rat P1-3 SC Cereb (target) Ctx | Rat E14-18 SC SC SC Hip Hip | | 132.6±7.7 µ ¹ 575.9±40.6 µ 112.2±6.5 µ | 25% ² of neurons have 4 or more dendrites on SC or Cereb astroglia | 23.8±1 µ ¹ 25.8±1 µ | Target derived astroglia increase axon length. Cell-cell contact is not necessary. |
| | | Hip Ctx (target) | | | 146.5±7.5 µ 276.8±11.5 µ | 20% ² of neurons have 4 or more dendrites on Ctx or Hip astroglia | 38.6±1.8 µ 42.2±2.2 µ | Dendrite outgrowth is not influenced by astroglia in a neuroanatomic specific manner. |
| Rousselet et al 1990 | 2 DIV Toluidine Blue (NF-200) (MAP2) | Rat E14 Mes CM Str CM | Rat E14 Mes Mes | 3.5 neurites ² per neuron, total length 255.8±5.3 µ, longest 116.8±3.7µ ¹ 2 neurites ² per neuron, total length 209.8±8.0 µ, longest 173.2±5.7µ ¹ | | | | Increased number of primary neurites initiated in homotypic astroglial medium. |

| AUTHOR | ANALYSIS | ASTROGLIA | NEURON | NEURITE #/L | AXON | DENDRITE # | DENDRITE L | CONCLUSIONS |
|--------------------------------|----------------------------------|--------------------------------|--------------------------------|---|------|--|------------|---|
| Rousselet et al 1988 | 2 DIV Toluidine Blue NF | Rat E14-16 Mes CM Str CM | Rat E14-16 Mes Mes | 57% ² neurites ≥4neurites 30% neurons ≥4neurites Total neurite length Mes CM > Str CM, differed by < 20% | | | | 'Dendrite' like neurites preferentially initiated in homotypic astroglial medium. |
| Denis-Donini & Estenoz 1988 | 3 DIV 3H DA -AR | Mouse OB Mes Str | Mouse E13 Mes Mes Mes | Total neurite output ¹ 2450 ± 900 μ 1347 ± 320 μ 725 ± 210 μ | | | | Longest neurite on non homotypic, non target astroglia. Cell-cell contact required. No neuroanatomic selective astroglial influence on OB neurons observed. |
| Autillo et al 1988 | 2 DIV NF an | Mouse E15 Mes Str | Mouse E15 Mes Mes | 47% ² neurites ≥3neurites 15% ² neurons ≥3neurites | | | | Increased primary neurites and branches on homotypic astroglia. Length not analyzed. |
| Chamak et al 1987 | 2 DIV MAP2 | Rat E15 Mes Str | Rat E15 Mes Mes | | | 40% ² neurons ≥ 3 MAP2-IR neurites (mean ±2) 20% ² neurons ≥ 3 MAP2-IR neurites (mean ±1.5) | | Increased primary neurites and branches on homotypic astroglia. Length not analyzed. A similar but less pronounced effect was observed for Str neurons cultured on Mes or Str astroglia. |
| Denis-Donini et al 1984 | 1,2,3,10 DIV 3H-DA AR | Mouse E13-15 Mes Str | Mouse E13 Mes Mes | 3DIV 74% ¹ neurons branched 0% neurons branched | | | | Increased # primary neurites observed on homotypic astroglia. |

Table 3: The table summarizes the available literature addressing the role of astroglia from different CNS regions on CNS neuron process outgrowth. Several points can be made. In general, homotypic astroglia appear to be the best stimulant of dendrite growth from neurons; however, certain astroglial subtypes, for example retinal and olfactory bulb may also be able to promote dendrite growth. Second, the astroglial effect on dendrite growth is primarily on process number rather than length, but in most cases length has not been specifically analyzed. Third, insufficient data exists to determine whether certain astroglia are consistently good or poor promoters of dendrite growth. Finally, target derived astroglia may increase axon length; however, certain astroglial subtypes, such as those derived from olfactory bulb may also stimulate axon growth. (Abbreviations: 1= data reported in the paper, 2 = an estimate derived from tables or figures reported in the paper, DIV = days *in vitro*, SC = spinal cord, Mes = mesencephalon, Str = striatum, OB = olfactory bulb, Ret = retina, Ctx = cortex, Hip = Hippocampus, Cereb = Cerebellum CM = conditioned medium, em = electron microscopy, IR = immunoreactive, 3H DA-AR = Dopamine autoradiography, # = number, L = length, + = positive stimulus).

different areas might instead be their relative ability to support axon or dendrite growth. Furthermore, it is not known whether glia from different CNS regions specifically influence cortical neurons in a selective neuroanatomic manner. We, therefore decided to test this hypothesis by examining neuron process outgrowth from cortical neurons in the presence of astroglia from a wide variety of CNS sources. Both axonal and dendritic growth from mouse cortical neurons in culture were quantified using double-labeled immunohistochemical techniques. Neurons were plated at low density and isolated neurons that were not in contact with other neurons were analyzed, since there is evidence that increased neuron density (Chamak et al 1987, Bruckenstein and Higgins 1988, Mattson et al 1989), synaptic activity (Rubel et al 1981, Deitch & Rubel 1984, Schilling et al 1991), the presence of axons and the local application of neurotransmitters (Mattson et al 1988a, 1988b, and 1989) can all influence neurite growth. The data reported here demonstrate that while astroglia are generally permissive for neurite growth, astroglia from different regions of the CNS differ in their ability to support dendritic, but not axonal growth from mouse cortical neurons *in vitro*. The ability to support extensive dendritic growth from cortical neurons, however, is not confined to either homotypic or target derived astroglia and in part, is due to the release of a diffusible factor.

MATERIALS AND METHODS

The experimental methods are described in detail in chapter 7. Briefly, cortical neurons were prepared from E18 mouse embryos and plated at low density on: 1) P4 rat astroglial monolayers prepared from various CNS regions including, cerebral cortex, mesencephalon, olfactory bulb, striatum, spinal

cord, or retina; or 2) poly-D-lysine coated coverslips in a noncontact coculture system in which the astroglia continuously conditioned the medium. The growth of axons and dendrites from cortical neurons, identified by M6, NF-H or MAP2 immunoreactivity, was quantified after 5 days *in vitro*. At least three experiments for each test substrate or cell monolayer were carried out. A total of 100 double-labeled, immunoreactive neurons were examined in each experimental condition as described in chapter 7.

RESULTS

Glial characterization.

There is increasing evidence that astrocytes are not a homogenous population (Hannsen 1988, Barres 1991, O'Malley et al 1992, Black et al 1993, Holzwarth et al 1994). Several lines of evidence suggest that astroglia from different regions of the CNS differ in their ability to promote neurite outgrowth (Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Denis-Donini and Estenoz 1988, Prochiantz et al 1990, Qian et al 1992). Table 3 summarizes these results. Two points are apparent. First, there have been relatively few studies that have quantitatively examined the effects of glia from different CNS regions on both axon and dendrite growth. Second, there is insufficient information to conclude whether certain glia are in general better promoters of dendrite growth than other glia. Taken together these studies suggest that homotypic astroglia are more effective at promoting dendrite growth whereas target derived astroglia are more effective promoting axon growth. The results of some experiments, however, suggest that astroglia derived from particular CNS regions, for example olfactory bulb, are generally more effective promoters of neurite growth. Therefore, in the present study,

astroglial cells were derived from a wide variety of different CNS regions including, cortex, striatum, mesencephalon, spinal cord, olfactory bulb, and retina from early postnatal (P4) rats. Cells were maintained for approximately 10 days in serum containing medium and between 2 and 5 days in chemically defined medium. Glia, when cultured in this manner, contained few, if any neurons (Figure 6). MAP2 and NF-H immunoreactivity was not detected in the glial monolayers. To characterize the cellular composition of the monolayers, cultures were stained with a nuclear stain, 4', 6-diamido-2-phenyl indole (DAPI; Sigma) and cell type specific antibodies. The number of immunoreactive cells were then calculated as a percentage of the total number of cells present. Immunohistochemical characterization demonstrated that the majority of the cells (83.5%-89.5%) could be classified as type I astrocytes based on morphology and GFAP immunoreactivity (Table 4). Most cells exhibited a flat, polygonal morphology (Figure 7). Some isolated astroglial cells were multipolar and occasional, labeled cells were stellate in morphology. In most of the monolayers, few cells had the immunohistochemical phenotype of type II astrocytes (GFAP+; A2B5+); in the cortical cultures, 3.7% of the cells could be classified as type II astrocytes on the basis of these antigens. Staining with galactocerebroside and Thy 1.1 demonstrated few oligodendrocytes and fibroblasts, respectively, in any of the glial monolayers.

The immunohistochemical and morphological characteristics of the monolayers were very similar for astroglia derived from most CNS regions examined (Table 4). Whereas retinal and olfactory bulb astroglia were morphologically similar to astroglia from other regions, they demonstrated immunohistochemical differences. The majority of cells in the retinal

FIGURE 6: Purified astroglial cultures were prepared from several regions of the rat brain as described in Materials and Methods. Representative phase contrast photomicrographs of developing cortical glial cultures (a-d) and fluorescent photomicrograph of purified astroglial monolayer labeled with anti-GFAP (e). After 3 days in culture (a), scattered astroglia and aggregates of neurons growing on top of the astroglia are visible. Flat polygonal type 1 astrocytes proliferate in serum; it is difficult, however, to distinguish astroglia from fibroblasts using phase contrast microscopy after 6 days *in vitro* (b). A few process bearing cells and small bipolar cells, that probably are type O2A astroglial progenitors, are spread randomly over the flat cells (b). The polygonal flat cells form a confluent monolayer after 10 days in culture; individual cells, however, lack polarity (c). Most neurons have died by now; process bearing cells, microglia and occasional neurons are clustered above the confluent astroglia (c). Loosely adherent cells found growing on top of astroglia are removed by overnight shaking and gentle trypsin treatment (d). The vast majority of remaining attached cells exhibit a fairly uniform cobblestone appearance and represent type 1 astrocytes as demonstrated by GFAP immunoreactivity (e). (Scale bar = 10 μ m).

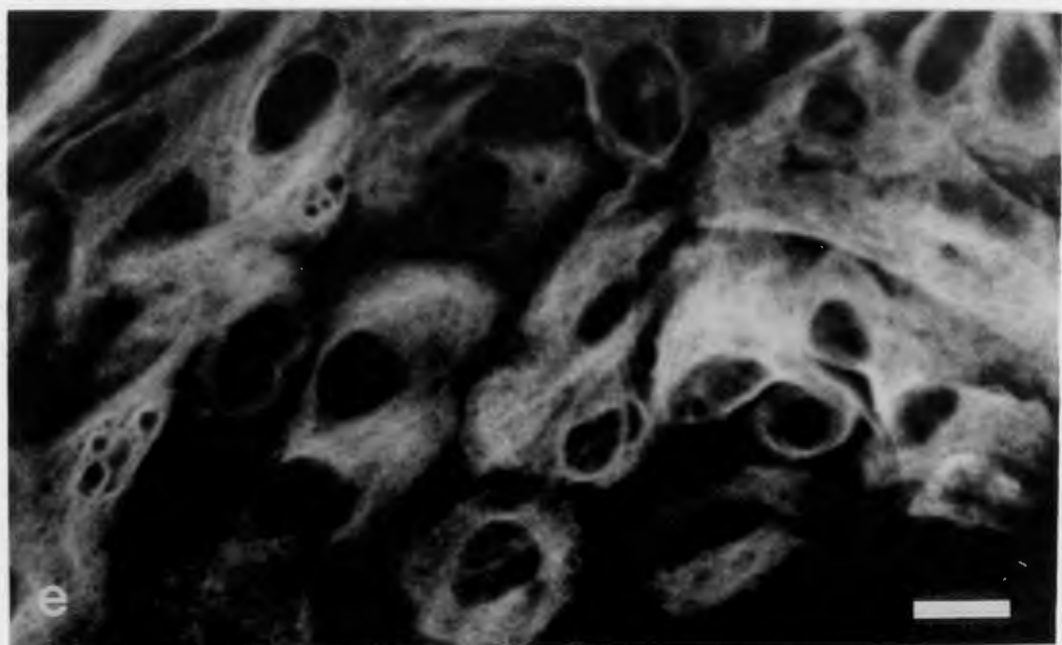
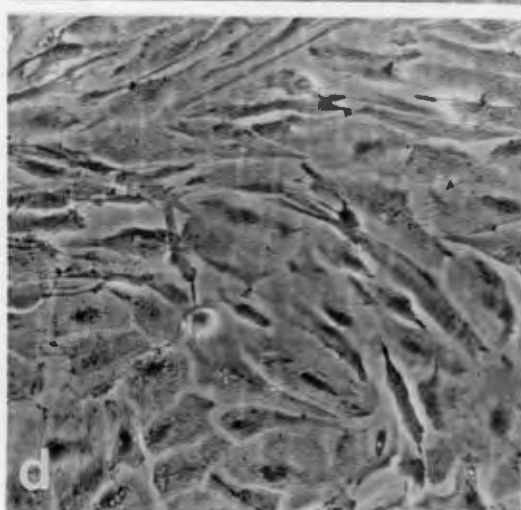
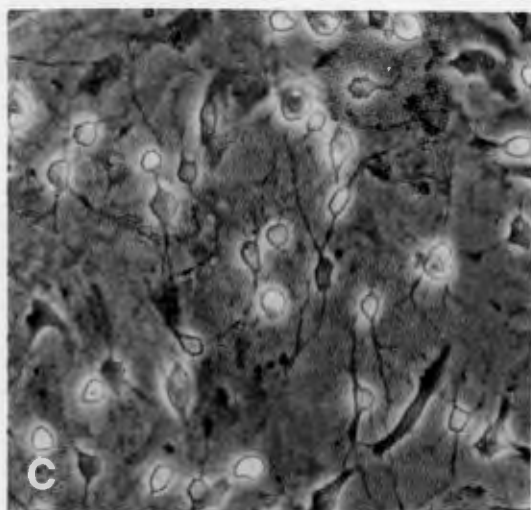
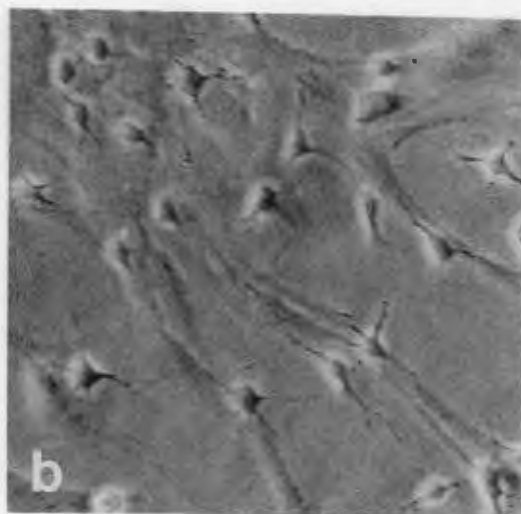


FIGURE 7: Astroglial monolayers were prepared from different CNS regions as described in Materials and Methods. Representative fluorescent photomicrographs of astroglial monolayers derived from cortex (a and b) and mesencephalon (c and d), labeled with anti-GFAP (a and c) and DAPI (b and d). The majority of cells demonstrated a flat polyglonal morphology and could be classified as type 1 astrocytes. (Scale bar = 10 μm)

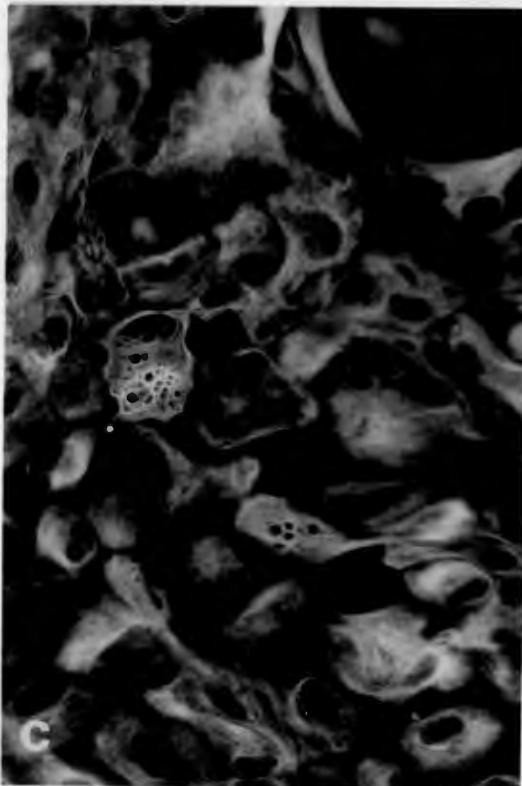
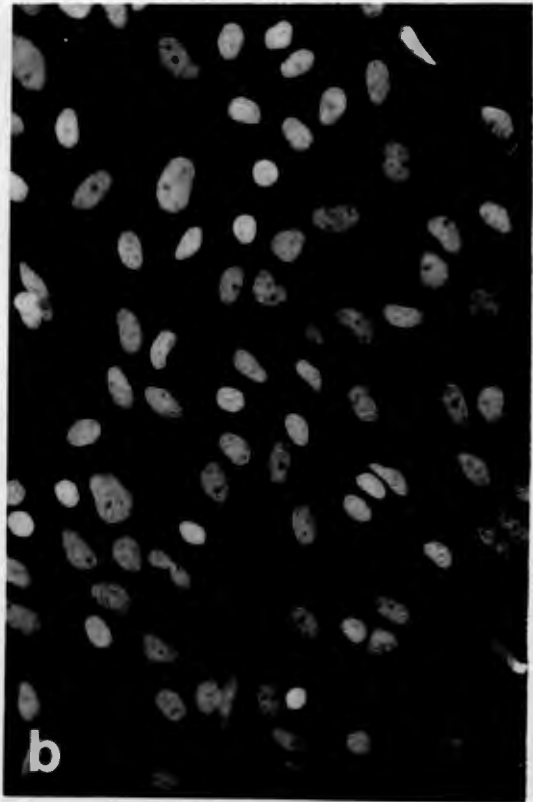
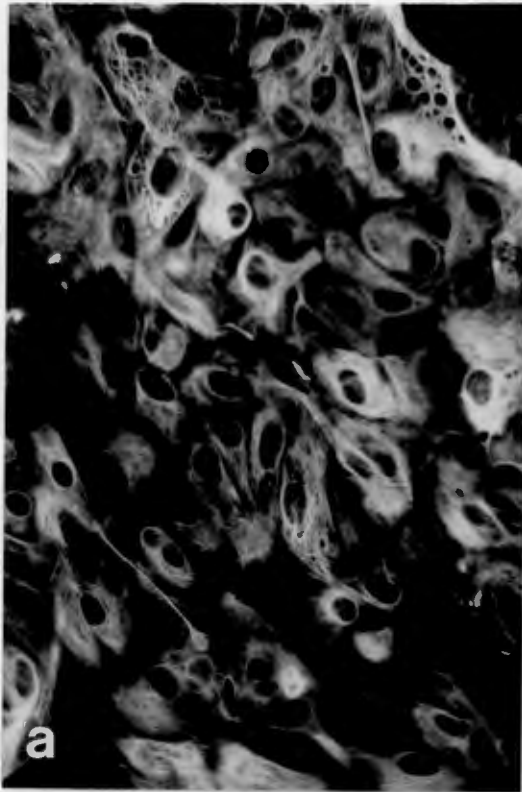


TABLE 4: CELLULAR COMPOSITION OF ASTROGLIAL MONOLAYERS

| Cell type | Antigenic marker | FIB | SC | MES | STR | OB | RET | CTX |
|-------------------|------------------|------|------|------|------|------|------|------|
| Type I astrocyte | GFAP+/A2B5- | - | 83.5 | 83.5 | 86.4 | 33.7 | - | 89.5 |
| Type II astrocyte | GFAP+/A2B5+ | - | 0.5 | 2 | 1 | - | - | 3.7 |
| O2A progenitor | GFAP-/A2B5+ | - | - | - | - | - | - | - |
| Oligodendrocyte | GFAP-/GAL-C+ | - | 1.5 | 1.5 | 1 | - | - | 2.3 |
| Fibroblast | Thy 1.1 + | 92.6 | - | 4.2 | - | 1.7 | 0.25 | 2.2 |
| Neuron | MAP2+/NF+ | - | - | - | - | - | - | - |
| Müller Glia | VM+/CRABLP+ | - | - | - | - | - | 86.5 | - |
| Ensheathing glia | GFAP+/NGFR+ | - | - | - | - | 14 | - | - |
| Immature glia | VM+ | - | 18 | 53 | 82 | 88 | - | 83.5 |
| Progenitor | RAT 401+ | - | - | - | - | - | - | - |
| Mouse contaminant | M2+/M6+ | - | - | - | - | - | - | - |

Table 4: Astroglial cells were derived from early postnatal (P4) rat CNS regions as described in Materials and Methods and characterized with cell specific antibodies. Data are expressed as the percentage of total cells identified by a nuclear stain (DAPI) that are immunoreactive for a specific antibody (- indicates not detected). Five random fields from five coverslips of each monolayer were examined from 3 independent cultures. The total number of cells counted for each CNS region ranged from 750-1000. Immunohistochemical characterization indicates: 1) the complete absence of neurons and 2) the majority of cells are either GFAP or Vimentin positive and therefore represent astroglia at various maturational stages. (Fib = fibroblasts from meninges, SC = spinal cord, Mes = mesencephalon, Str = striatum, OB = olfactory bulb, Ret = retina, Ctx = cortex)

astroglia cultures expressed vimentin and cellular retinaldehyde binding protein (CRALBP). The latter antibody is expressed specifically by Müller glia in the sensory retina (de Leeuw et al 1990). In the olfactory bulb, the following distinct cell populations could be distinguished; 1) flat cells with a typical astrocyte morphology, that were immunoreactive for vimentin, and 2) elongated cells, with fairly long processes that extended across the surface of other cells. Although these latter cells resembled the O2A progenitors (Raff 1989), immunoreactivity for A2B5 was not detected. By contrast, these cells expressed GFAP and the low affinity NGF receptor, as detected by the monoclonal antibody 192, and may represent olfactory ensheathing glia (Doucette 1990, Vickland et al 1991). The majority of cells in the cortex and striatum as well as many cells in the mesencephalon, expressed both GFAP and vimentin. By contrast, very few spinal cord astroglia expressed both antigens, while most olfactory bulb and retinal astroglia displayed vimentin immunoreactivity only. No immunoreactivity was observed for Nestin (RAT 401) in any of the cell populations examined. Vimentin cross reactivity was not observed with Thy 1-1 or neurons that expressed NF-H or MAP2.

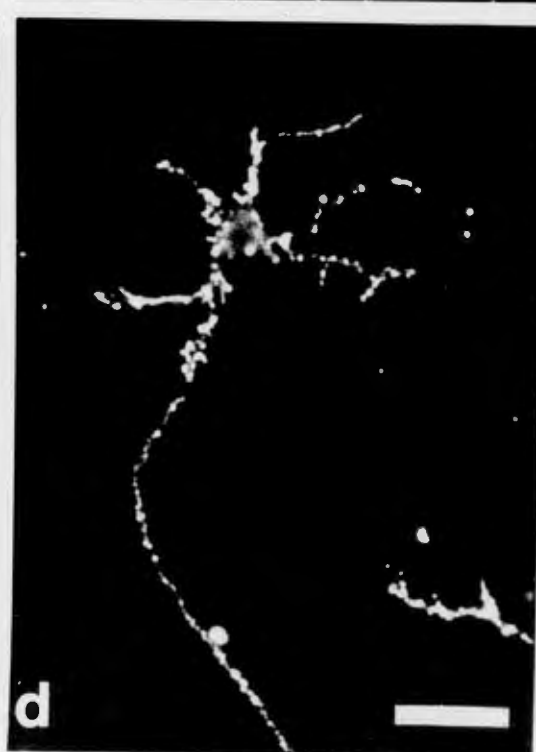
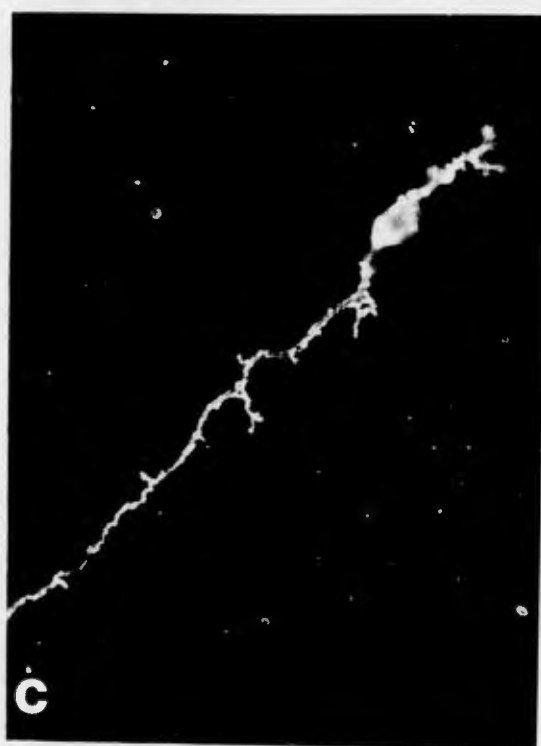
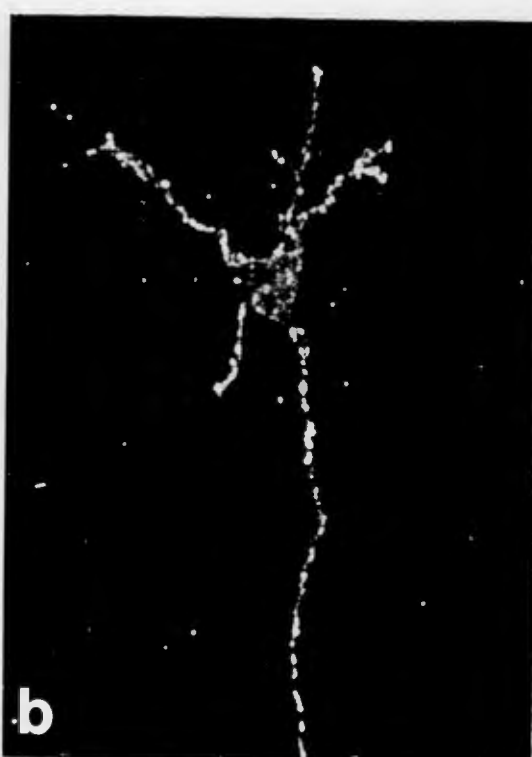
Dendritic growth is selectively influenced by the anatomic source of the astroglial monolayer.

We analyzed the potential influence of astroglia from different CNS regions on cortical neuron dendrite growth. In order to identify the full extent of the processes from cortical neurons, embryonic (E18) mouse cortical neurons were cocultured onto rat astroglial monolayers and the antibody M6 was used to selectively label the mouse neurons (Lund et al 1985, Lagenaur et al 1992). The M6 antigen was distributed on all processes of the neurons with patches of continuous labeling alternating with punctate staining. As we were

principally interested in the effect of astroglia on dendrite growth, we plated neurons at low density (1×10^4 cells/ml) and only analyzed isolated neurons; i.e. those not in contact with other neurons, that were double immunoreactive for M6 and MAP2 or NF-H. Neurite outgrowth was analyzed after 5 days *in vitro* to allow for the selective localization of MAP2 and NF-H to dendrites and axons, respectively.

Figure 8 illustrates the main differences observed in the morphologic aspects of cortical neurons grown for 5 days *in vitro* on different glial monolayers. On monolayers generated from cortex, retina and olfactory bulb; most neurons were round, multipolar and extended one long process and several shorter processes. By contrast, neurons grown on striatal, mesencephalic or spinal cord astroglia exhibited a unipolar or bipolar morphology. The neuronal processes were characterized using antibodies directed against NF-H and MAP2 to identify axon and dendrites respectively. The results of this analysis are illustrated in figure 9 and table 5. On cortical or retinal glial monolayers, the cortical neurons had an average of three or more primary dendrites. By contrast, almost all neurons (86.4% -100%) grown on striatal, mesencephalic or spinal cord astroglia extended only one or occasionally two MAP2 immunoreactive processes (Table 5). Between 56.2% and 81.7% of primary dendrites were greater than $10\mu\text{m}$ in length. With the exception of striatal astroglia, the length of individual primary dendrites did not significantly differ on the different astroglial monolayers (Figure 9). When neurons were grown on astroglia from the striatum, only 12% of primary dendrites achieved lengths greater than $20\mu\text{m}$. By contrast, 25.5% - 42.9% of primary dendrites examined on other monolayers had lengths greater than $20\mu\text{m}$. Dendrites tapered and grew directly from the cell body in a relatively straight direction.

FIGURE 8: Cortical neurons growing on different glial monolayers *in vitro* exhibit striking dendritic differences dependent on the CNS region from which the cells are derived. Representative fluorescent photomicrographs of E18 mouse cortical neurons labeled with anti-M6 after 5 days *in vitro* (a-f). Whereas axonal growth was similar on all monolayers tested, on glial monolayers derived from spinal cord, striatum or mesencephalon (a, c, e), neurons do not demonstrate an extensive dendritic arbor and usually exhibit a uni- or bipolar morphology. By contrast, on glial monolayers generated from the retina, olfactory bulb or cortex (b, d, f), most neurons are multipolar and extend an axon and several dendrites. Neurons grown on polylysine coated coverslips in continuously conditioned astroglial medium, exhibit similar morphologic differences depending on the CNS source of the astroglia. Lower power micrographs of E18 mouse cortical neurons grown on astroglial monolayers derived from mesencephalon (e) and cerebral cortex (f) demonstrate that although the axons are of similar length (arrowheads), the neuron growing on the cortical astrocytes has four dendrites extending from the cell soma, whereas the neuron growing on mesencephalic astrocytes has only two dendrites (arrows). (Scale bar = 10 μ m)



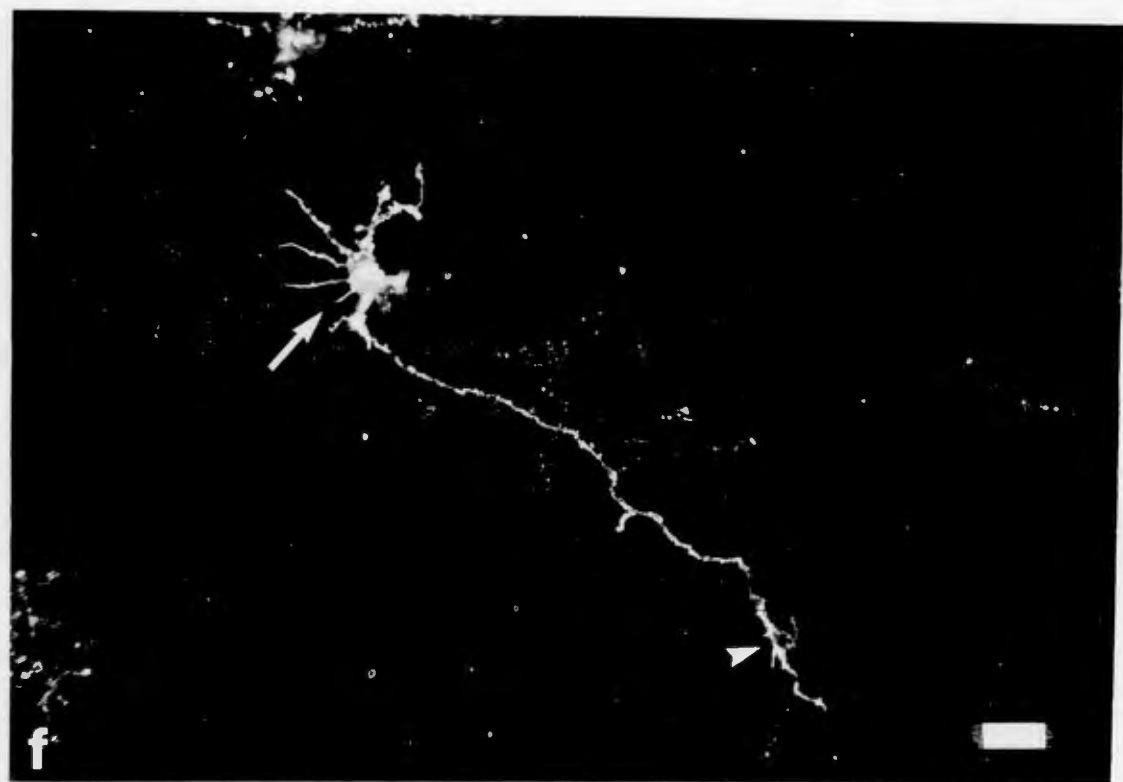
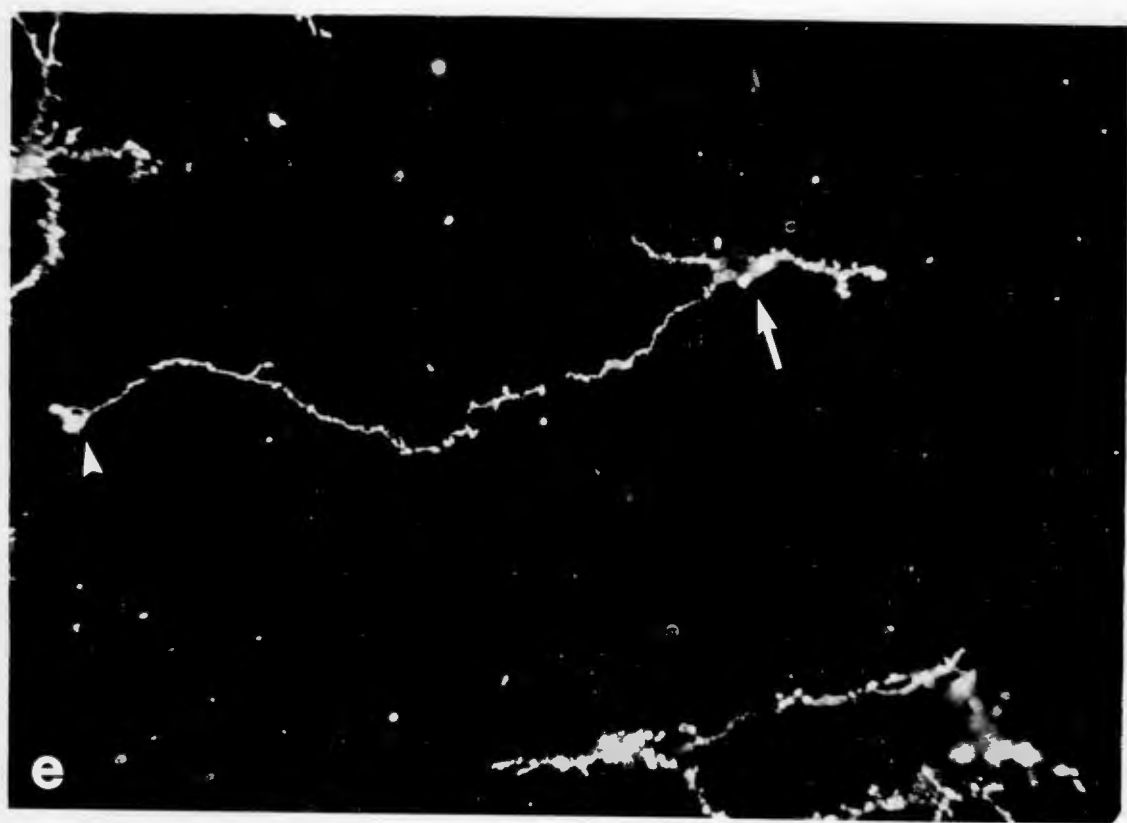
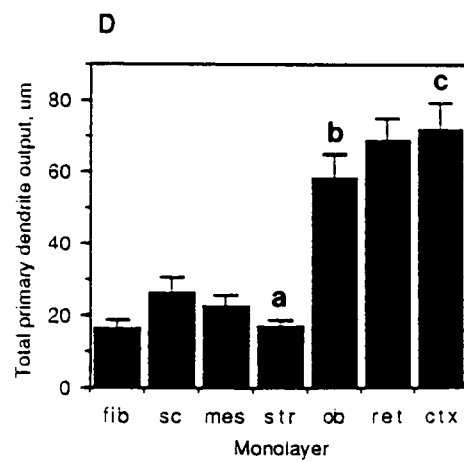
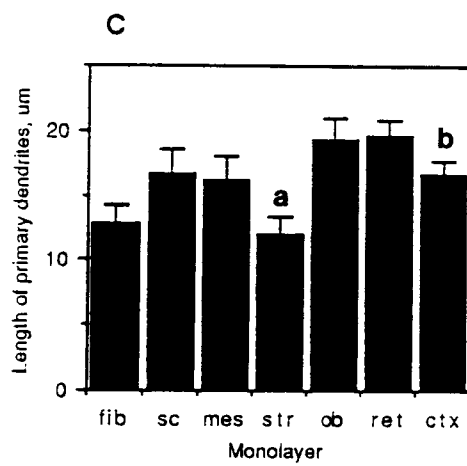
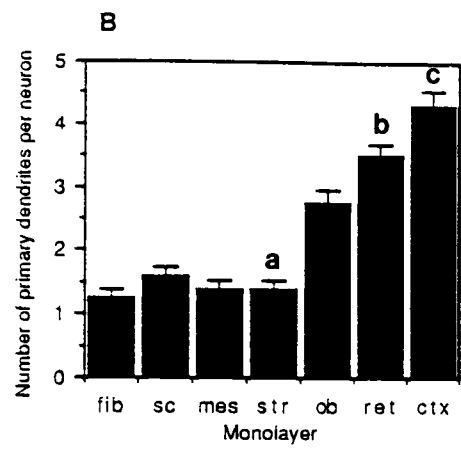
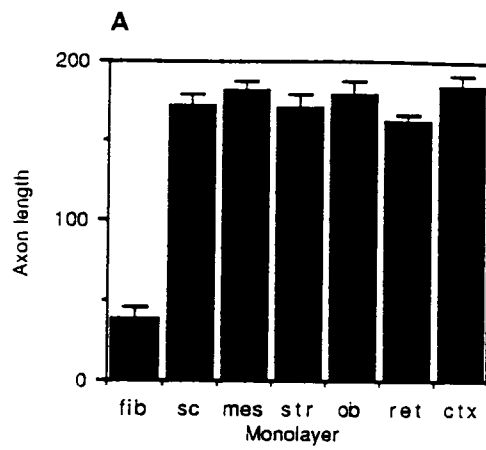


TABLE 5: THE NUMBER OF PRIMARY DENDRITES ELABORATED BY CORTICAL NEURONS DEPENDS ON THE ORIGIN OF THE ASTROGLIA.

| MONOLAYER | % MAP ₂ + neurites | | | | | |
|-----------|-------------------------------|------|------|------|------|------|
| | 0 | 1 | 2 | 3 | 4 | > 4 |
| FIB cc | 14.3 | 47.6 | 38.1 | 0 | 0 | 0 |
| FIB mp | 23.8 | 61.9 | 9.5 | 0 | 4.8 | 0 |
| SC cc | 0 | 54.5 | 31.8 | 13.6 | 0 | 0 |
| SC mp | 3.7 | 53.3 | 30.7 | 3.8 | 0 | 0 |
| MES cc | 0 | 65.4 | 26.6 | 6.6 | 0 | 0 |
| MES mp | 13.3 | 53.3 | 26.6 | 6.6 | 0 | 0 |
| STR cc | 0 | 66.6 | 33.3 | 0 | 0 | 0 |
| STR mp | 0 | 81.3 | 18.8 | 0 | 0 | 0 |
| OB cc | 0 | 9.1 | 36.4 | 31.8 | 13.6 | 9.1 |
| OB mp | 0 | 37.5 | 37.5 | 12.5 | 12.5 | 0 |
| RET cc | 0 | 0 | 8.3 | 45.8 | 33.3 | 12.5 |
| RET mp | 5.9 | 5.88 | 11.8 | 23.5 | 41.2 | 11.8 |
| CTX cc | 0 | 0 | 4.3 | 8.7 | 60.8 | 26.1 |
| CTX mp | 0 | 5.6 | 22.2 | 33.3 | 22.2 | 16.7 |

Table 5: Mouse cortical neurons were cocultured with astroglia from different CNS regions as described in Materials and Methods. After 5 DIV the number of primary MAP2 immunoreactive processes elaborated from 100 random, isolated cortical neurons on each monolayer were examined. The results represent the percentages of neurons with a specified number of MAP2 positive neurites. Although the most primary dendrites were observed on cortical astroglia, the number of primary dendrites were also observed to significantly increase on retinal and olfactory bulb astroglia. (0 to >4 represent the number of MAP2 positive neurites elaborated by neurons; mp = noncontact coculture, cc = direct coculture, Fib = fibroblasts from meninges, SC = spinal cord, Mes = mesencephalon, Str = striatum, OB = olfactory bulb, Ret = retina, Ctx = cortex)

FIGURE 9: Cortical neuron morphogenesis can be selectively influenced by astroglial cells in culture. Histograms illustrating axon length (a), primary dendrite number (b), primary dendrite length (c) and total dendrite output (d) from mouse cortical neurons cocultured with rat glia derived from different CNS regions as described in Materials and Methods. Neurite elaboration was examined in 100 randomly selected neurons growing on each glial monolayer using quantitative analysis and double-labeling immunohistochemical techniques. Values represent the mean \pm SEM. Fib included as control for Students t -test but not for ANOVA. Whereas axon length was similar on all monolayers tested (ANOVA $p > 0.05$), total output of primary dendrites was threefold greater on astroglia derived from cortex, retina or olfactory bulb than for astroglia from other CNS regions (ANOVA $p < 0.001$; a = $p > 0.05$ vs fib, mes, sc; b = $p < 0.0001$ vs fib, sc, mes, str; c = $p > 0.1$ vs ob, ret). An increase in the number of primary dendrites (ANOVA $p < 0.001$; a = $p > 0.1$ vs fib, mes, sc; b = $p < 0.0001$ vs fib, sc, mes, str; c = $p < 0.01$ vs ob, ret) and to a lesser extent, elongation of individual dendrites (a = $p > 0.05$ vs fib, sc, mes, ctx and $p < 0.01$ vs ob, ret; b = $p > 0.05$ vs fib, sc, ret) observed from cortical neurons was dependent on the source of astroglial cells. (Fib = fibroblasts from meninges, SC = spinal cord, Mes = mesencephalon, Str = striatum, OB = olfactory bulb, Ret = retina, Ctx = cortex)



Branch points were seen from only a few primary dendrites particularly those growing on cortical or retinal monolayers or when contact had been made with other neurons. Too few branch points were demonstrated to quantify this observation.

In order to characterize the influence of astroglia on primary dendrite output further, the total extent of the primary dendritic arbor was quantified. The results indicated that 21.7% of the cortical neurons cocultured with cortical astrocytes had primary dendritic arbors with lengths greater than 100 μ m. Extensive primary dendrite elaboration was also observed from cortical neurons that were cocultured with olfactory bulb or retinal astroglia; 12% and 16.7% of neurons achieved arbors greater than 100 μ m respectively. By contrast, total primary dendritic elaboration only exceeded 50 μ m on 8%, 0% and 13.6% of mesencephalic, striatal or spinal cord astroglial monolayers, respectively (Figure 9). Mean total primary dendritic outgrowth was greatest on astroglia derived from cortex, olfactory bulb or retina and was nearly threefold greater than that observed on astroglia from striatum, mesencephalon or spinal cord (ANOVA $p < 0.001$; Figure 9). These observations indicate that astroglia demonstrate regional differences, although not exclusively region or target specific, in their ability to support cortical neuron dendrogenesis. This effect is principally on dendrite number and total dendrite output rather, than on elongation of individual dendrites.

Axon length does not differ on astroglial cells from diverse CNS regions.

Antibodies directed against NF-H stained only one neurite emerging from the cell body. The process with NF-H immunoreactivity was usually the longest process in both multipolar and bipolar cells. In unipolar cells, the cell body

expressed MAP2 whereas the process was usually immunoreactive only for NF-H. Occasionally, neurites were observed to be immunoreactive for both MAP2 (proximally) and NF-H (along the entire length of the process). These neurites were classified as axons. Since the mouse neurons were plated at low density, the entire length of individual axons could be followed and quantified. Axons tended to follow a meandering course from the cell body and virtually all axons had a growth cone at their tips. Axonal length of cortical neurons did not differ significantly when plated on the various glial monolayers; 86.8% -100% of axons examined were greater than 130 μm and 38% -61.5% were greater than 170 μm in length. There was a tendency for greater axon lengths from cells cultured on cortical astroglia; however the difference was not statistically significant, (ANOVA $p>0.05$; Figure 9). The shortest axons were observed on Müller glia; less than 10% achieved lengths greater than 200 μm whereas on other astroglia, between 18.2% and 35.2% of axons were greater than 200 μm in length.

These immunohistochemical data indicate that astroglial cells derived from cortex, retina and olfactory bulb allowed initiation and growth of axons and dendrites from mouse cortical neurons *in vitro*, while astroglial cells derived from striatum, mesencephalon and spinal cord supported axon growth, but only limited dendrite elaboration.

Diffusible factors released by astroglia may account for part of the regional differences observed in dendrite growth.

Do the observed differences in the capabilities of astroglia to support dendrite growth reflect specific cell-surface interactions, or differences in soluble factors released by astroglia? In the next series of experiments, we compared the

number and length of neurites from cortical neurons grown on glial monolayers with those grown in a noncontact coculture system in which the astroglia continuously conditioned the medium. Embryonic mouse cortical neurons were plated at low density (1×10^4 cells/ml) directly onto polylysine coverslips in a chemically defined medium. Astroglial cells derived from different CNS regions were grown on a millipore filter above and separate from the polylysine coverslips.

After 5 days *in vitro*, neurons exhibited regional morphological differences similar to the differences that were described in the previous section. The majority of neurons grown in the presence of cortical, retinal or olfactory bulb astroglia were multipolar whereas neurons grown under spinal cord, mesencephalon or striatal glial monolayers were typically unipolar or bipolar. Significant differences in the number of primary dendrites from cortical neurons were observed depending on the CNS source of the astroglia, (ANOVA $p < 0.001$; Table 5 and Figure 10). In medium conditioned by cortical or retinal astroglia, 72.2% and 76.5% respectively, of neurons examined had 3 or more dendrites. By contrast 0%, 6.6% and 3.8% of neurons grown in medium conditioned by striatal, mesencephalon or spinal cord astroglia respectively, elaborated three or more dendrites. Olfactory bulb astroglial conditioned medium had an intermediate effect, 25% of neurons demonstrated three or more dendrites. Interestingly, the length of individual primary dendrites from cortical neurons did not differ significantly between the different glial monolayers; however an average 15%-25% reduction was observed when dendrite length in conditioned medium was compared to dendrite length in direct coculture. Although total primary dendritic elaboration was less profuse in continuously conditioned medium than in

direct coculture, regional heterogeneity was still observed. The greatest amount of primary dendritic growth was observed in medium continuously conditioned by astroglia derived from cortex, retina and to a lesser extent olfactory bulb (ANOVA $p < 0.001$; Figure 10). Taken together these observations suggest that astroglial cells may release factors into the culture medium, which are both generally permissive for neurite growth but can also specifically initiate and maintain primary dendrite growth.

Analysis of neuron survival and morphology.

A critical point in the interpretation of our results is possible selective survival or selection of a subpopulation of neurons, rather than astroglial stimulated neurite outgrowth. To determine whether this is a relevant factor, we assessed neuron survival at 1, 3 and 5 days *in vitro* using both morphological and immunohistochemical techniques. The number of viable neurons were expressed as a percentage of the number of viable cells that were initially plated. Six random microscopic fields on five coverslips were examined in three separate cultures of each cellular subtype. In order to avoid ambiguity, the neuronal cell types were confirmed by immunostaining with the antibody to MAP2. Immediately after plating at least 500 cells were counted for each monolayer; at each subsequent time point greater than 200 cells were examined. Viable cells had smooth, round to oval somata and neurites that appeared uniform in diameter and smooth in appearance. Non-viable cells had rough, swollen and vacuolated somata and irregular, fragmented or beaded neurites. Although there was some variation in neuron survival at 5 days *in vitro* among the monolayers prepared from different CNS regions; from 41% in striatum and retina to 48% for olfactory bulb and spinal cord (ANOVA $p < 0.001$; Figure 11); this variation, however,

FIGURE 10: Cortical neuron dendrite growth is, in part regulated by a diffusible factor released by astroglia. Mouse cortical neurons were grown in a noncontact coculture system in which the medium was continuously conditioned by glial monolayers derived from various CNS regions. After 5 DIV axon length (a), primary dendrite number (b), dendrite length (c) and total primary dendrite output (d) was quantified from 100 randomly selected neurons for each astroglial subtype. Results represent the mean \pm SEM. Fib included as control for Students t-test but not for ANOVA. Noncontact (mp) and direct coculture (cc) are compared. In continuously conditioned medium (mp), regional heterogeneity in astroglial mediated axon growth was not observed with the exception of olfactory bulb astroglia (a = $p < 0.0001$ vs fib; $p < 0.05$ vs sc, mes, str, ret, ctx; b = $p > 0.05$ vs sc, mes, str, ctx). By contrast, dendrite output depended on the region from which the astroglia were derived. Although primary dendrite elaboration was less prolific in continuously conditioned medium (mp) than direct coculture (cc), greater total primary dendrite output (ANOVA $p < 0.001$, a = $p < 0.05$ vs fib; $p < 0.01$ vs ob; $p > 0.5$ vs mes, str; b = $p < 0.05$ vs ret, ctx; c = $p > 0.05$ vs ctx) and in particular an increase in the number of primary dendrites (ANOVA $p < 0.001$; a = $p > 0.5$ vs fib, sc, mes; $p < 0.01$ vs ob; b = $p < 0.01$ vs ret, ctx) was still observed in the presence of cortical or retinal astroglia and to a lesser extent olfactory bulb astroglia. The length of individual dendrites (c), unlike direct coculture, did not demonstrate regional differences (ANOVA $p > 0.1$; a = $p < 0.001$ vs fib; $p > 0.05$ vs mes, str, ob, ret, ctx; b = $p > 0.05$ vs mes, ret). (Fib = fibroblasts from meninges, SC = spinal cord, Mes = mesencephalon, Str = striatum, OB = olfactory bulb, Ret = retina, Ctx = cortex)

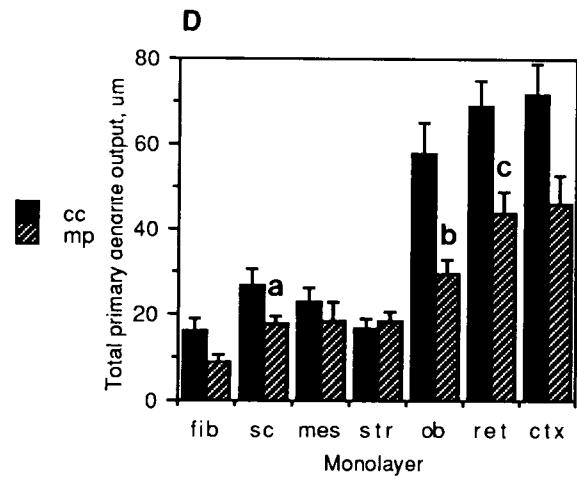
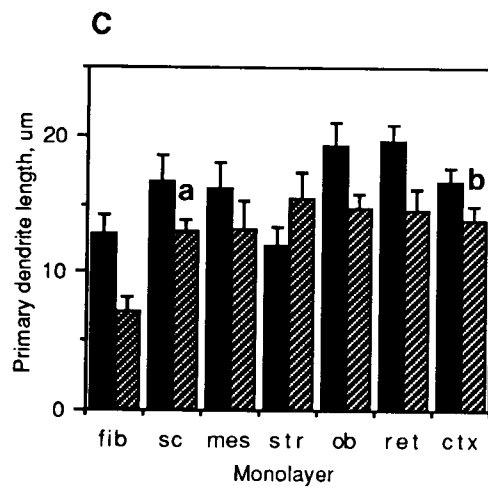
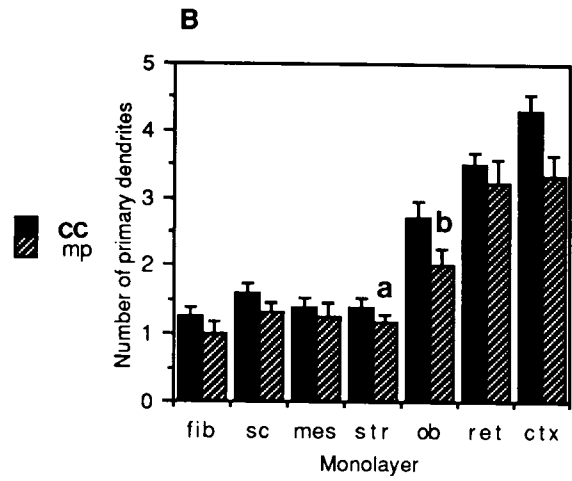
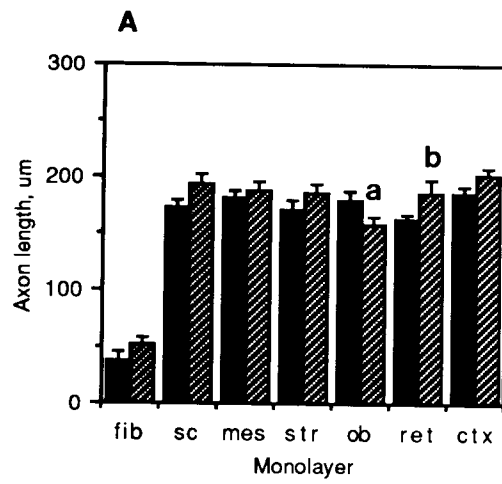
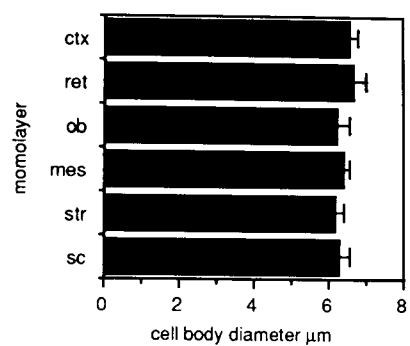


FIGURE 11: Neuron survival and cell body diameter. **A)** Cortical neuron survival varies among different glial monolayers but does not correlate with dendrite outgrowth. Neurons were plated at 1×10^4 cells/ml in the presence of astroglia derived from various regions of the CNS and neuron viability determined with morphologic and immunohistochemical techniques at 1, 3 and 5 DIV. Values for neuronal survival are expressed as the percentage of the original number of viable neurons at 3 hours in culture and represent the mean \pm SEM. Six random microscopic fields on 5 coverslips were examined in three separate cultures of each astroglial subtype. Fibroblasts and polylysine represent controls. Although astroglia had a clear effect on survival and variance was observed among the various astroglial subpopulations (ANOVA $p < 0.001$), the extent of dendritic elaboration was independent of this effect. **B)** Astroglia derived from different CNS regions do not select subpopulations of cortical neurons. Histograms illustrating mouse cortical neuron cell body diameter. Embryonic neurons were cocultured with astroglia for 5 days and examined after labeling with M6 or MAP2. Although there was a tendency for neurons with more dendrites to appear larger, quantitative analysis did not demonstrate a difference in neuron cell body diameter on the different glial monolayers tested (between group ANOVA $p > 0.1$). Values represent the mean \pm SEM ($n=100$ neurons for each glial subtype). (Fib = fibroblasts from meninges, SC = spinal cord, Mes = mesencephalon, Str = striatum, OB = olfactory bulb, Ret = retina, Ctx = cortex)

A

| Monolayer | 1 DIV | 3DIV | 5DIV |
|----------------|----------|----------|----------|
| Fibroblast | 68.6±3.3 | 52.9±5.6 | 38.8±2.5 |
| Spinal Cord | 81.6±2.8 | 66.5±3.1 | 48.1±2.7 |
| Mesencephalon | 81.1±4.7 | 64±3.6 | 43.8±3.2 |
| Striatum | 80.4±4.1 | 63.2±5.3 | 41.5±3.3 |
| Olfactory bulb | 80.8±2.7 | 59.6±3.2 | 48.4±4.3 |
| Retina | 82.4±4.7 | 63.3±3.7 | 41.7±4.3 |
| Cortex | 83.6±3.8 | 60.8±3.1 | 44.9±2.5 |

B



did not correlate with the observed differences in process growth. The results of these experiments correlated closely with estimates of neuron viability in living cultures in which survival was determined by examining the same marked microscopic fields of approximately 1mm^2 at successive timepoints during coculture experiments.

To determine whether astroglia from different CNS regions selectively promoted survival of particular neuronal subpopulations, such as interneurons or projection neurons, we compared the size and morphology of the cell body of neurons plated on the different glial monolayers. Although there was a range in neuronal diameter on each monolayer, mean cell body diameter was virtually identical on all monolayers (Figure 11). Similarly, no distinct difference in neuron cell body shape was observed. In addition, neurons on all the various monolayers demonstrated axons of similar length (see above). Taken together these results indicate that the effect on process outgrowth on the various glial monolayers was specific rather than mediated through selective survival of a distinct neuronal subpopulation or a selective influence on neuronal survival.

DISCUSSION

The data reported in these experiments are consistent with and extend previous observations that astroglia can selectively modulate the morphologic development of neurons in culture. First, dendrite number, total primary dendrite output and to a lesser degree, length of individual dendrites from mouse cortical neurons *in vitro* were greater when cortical neurons were plated on astroglia from cortex, retina and olfactory bulb than

when plated on astroglia derived from mesencephalon, striatum or spinal cord. Although homotypic astroglia are not the exclusive source for promotion of cortical neuron dendrite outgrowth, they are among the most effective glia for stimulating dendrite elaboration. Second, axonal growth was similar on all the glial monolayers that were tested. Striatal, mesencephalic, spinal cord and even cortical astroglia may be regarded as target astroglia for cortical neurons. Cortical neurons, however, do not project to either the retina or olfactory bulb; thus target astroglia alone do not appear to selectively promote axonal growth from cortical neurons *in vitro*. Third, neurons grown in medium conditioned by astroglia, exhibited regional morphological differences depending on the CNS source of the astroglia. We propose, therefore, that for mouse cortical neurons *in vitro*: 1) axonal and dendritic growth are separately regulated, 2) astroglia from different CNS regions differ in their ability to support the outgrowth of primary dendrites, though not in an exclusively homotypic manner, and 3) certain astroglia release a factor or factors that can specifically modulate primary dendrite outgrowth.

Astroglia influence the number of primary dendrites.

In what way do astroglia modulate neurite growth from cortical neurons? The results suggest the interesting possibility that astroglia can specifically regulate or maintain the number of primary dendrites that are elaborated by neurons. Although *in vitro* studies have demonstrated that neurons may have an intrinsic ability to initiate dendrites and axons (Bartlett and Banker 1984a and 1984b), other studies have provided evidence that dendritic growth of a neuron can be influenced by interactions with other cells. *In vitro* studies of both the autonomic nervous system and CNS indicate that differences in glial modulation of neurite outgrowth, are primarily manifested in the

number of neurites elaborated and, to a lesser extent, in the degree of branching or elongation (Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Rousselet et al 1988, Tropea et al 1988, Johnson et al 1989, Rousselet et al 1990, Qian et al 1992). The final form that dendrites develop, including their secondary and tertiary branches may also be supported by astroglia but, in part, may be further influenced by neuronal activity (Wise et al 1979, Hammer et al 1981, Mattson et al 1988a, Petit et al 1988, Schilling et al 1991, Sanes et al 1992a).

This selective promotion of dendrite number has been argued as a basis for homotypic influences of astroglia on neurons. For example, Chamak et al (1987) observed an increase in the number of MAP2 immunoreactive neurites from mesencephalic neurons in the presence of homotypic astroglia than when cocultured with striatal astroglia. The change in the mean number of neurites per neuron, however, although significant, was small; approximately 1.5 dendrites per neuron on striatal astroglia versus 2 on mesencephalic astroglia. It is possible, therefore, that the differences observed by Chamak et al (1987) may, in part, be explained by the relative abilities of striatal or mesencephalic astroglia to support dendrite growth, rather than a specific homotypic influence. In addition, other studies have failed to find an exclusively homotypic pattern of astroglial-mediated neurite outgrowth. For example, Qian et al (1992) reported that the length and number of primary dendrites from spinal cord neurons were similar, when cocultured with either spinal cord or cerebellar astroglia. Our results confirm that there are substantial regional differences in the ability of astroglia to promote dendritic outgrowth, but that these differences do not follow exclusively homotypic lines.

Could the maturational state of astroglial cells in the various monolayers account for our observations? Experiments both *in vitro* and *in vivo*, indicate that mature astrocytes have a reduced capacity to support neurite, but not specifically dendrite, growth when compared with immature astrocytes (Smith et al 1986 and 1990). The relative degree of astrocyte maturation is likely to vary in different regions of the CNS at any particular developmental time (Edwards et al 1990, Landry et al 1990). However, it is unlikely that this is the sole explanation for the differences we have observed in the ability of astroglia to promote dendritic growth. Vimentin expression, typically expressed by immature astrocytes (Dahl 1981, Prochiantz et al 1982, Bovolenta et al 1984, Dupovey et al 1985, Frederickson and McKay 1988, Cameron and Rakic 1991), did not correlate with the ability of the astroglia to support dendrite growth. Although robust dendritic growth was observed on glial monolayers that contained a relatively high percentage of Vimentin immunoreactive cells, such as cortical astroglia, neurons plated on monolayers with a similar percentage of vimentin immunoreactive cells, such as striatal astroglia, had a limited dendritic arbor. A similar conclusion was reached by Chamak et al (1987) who did not find substantial differences in the number of MAP2 immunoreactive processes from embryonic mesencephalic neurons when cocultured with embryonic (E15 or E18) or postnatal astroglia (P4).

Astroglial origin does not determine axon length.

We have found that astroglia from all regions of the CNS that we tested support axon growth to a similar extent. This finding is, in part, consistent with previous investigations *in vitro* that indicate that axon growth appears

to continue even in conditions that do not support dendrite growth (Autillo-Touati et al 1988, Bruckenstein and Higgins 1988, Rousselet et al 1988 and 1990). In addition, other studies have found that astroglia are generally a favorable substrate for neurite outgrowth (Noble et al 1984, Fallon 1985).

There is some evidence that target derived astroglia may promote axonal growth. For example, Qian et al (1992) observed enhanced axonal growth when spinal cord neurons were cultured in the presence of, but not in direct contact with, cerebellar astroglia compared to astroglia from other CNS sources. Similarly, they observed enhanced axonal growth from hippocampal neurons cultured with cortical astroglia when compared to hippocampal astroglia. They concluded that target derived astroglia specifically promote axon growth and that axons are more sensitive than dendrites to factors released by astroglia. Some support for this contention is provided by Prochiantz and colleagues (Rousselet et al 1990). Although these investigators observed a more noticeable effect of homotypic astroglia on dendrite number, they also found that axon length was different depending on whether mesencephalic neurons were cultured with medium conditioned by striatal astroglia (mean length $173.2 \pm 5.7 \mu$) or mesencephalic astroglia (mean length $116.8 \pm 3.7 \mu$). This result is consistent with the proposal by Qian et al (1992) since the striatum is a target of certain neurons in the mesencephalon. In an earlier study, however, the same investigators (Rousselet et al 1988) observed that total neurite length from mesencephalic neurons was greatest in homotypic and not target derived (striatal) astroglial conditioned medium. The observation by Denis-Donini and Estenoz (1988), further complicates a simple interpretation. The primary mesencephalic neurons that project to the striatum are the dopaminergic neurons of the substantia nigra. These

investigators specifically examined this population of cells and observed that dopaminergic mesencephalic neurons had shorter neurites when cultured on striatal (target) astroglia than homotypic astroglia. The longest neurite outgrowth occurred on olfactory bulb astroglia, a non target, non-homotypic source of astroglia. Axons, however, were not specifically identified.

In this study, the retina and olfactory bulb can not be regarded as targets for cortical neurons, yet axon length was the same on these monolayers when compared to target derived astroglia such as striatum, mesencephalon or even spinal cord. Thus, for cortical neurons at least, astroglial heterogeneity and in particular target derived astroglia do not appear to selectively influence axon length *in vitro*. The apparent discrepancy between our results and those of Qian et al (1992) and Rousselet et al (1990) may, in part, be explained by our selection criteria. We only analyzed neurons that were not in synaptic contact with other neurons and may therefore have excluded axons from long projection neurons that are more likely to be in contact with other neurons. In addition, the small neuron cell bodies that we observed indicates that our sample may have included primarily non projection neurons. It is therefore possible that the astroglial factors that stimulate axon growth may be different for projection and non-projection neurons. This possibility has not been addressed by any study to date and needs further investigation.

Astroglial heterogeneity

Although no obvious morphologic differences were observed between glial monolayers derived from different CNS regions, there is increasing evidence that more subtle biochemical differences exist between the different astroglia.

Astrocytes from different anatomic regions have been found to express characteristic ion channel phenotypes, adhesive properties and neurotransmitter receptors (Hansson 1988, Barres 1991). Mesencephalic or striatal astroglia *in vitro* synthesize and express distinct glycoproteins on their surface (Barbin et al 1988). In addition, specific post-translational modifications or alternate mRNA splicing resulting in multiple isoforms of the same molecule may also play a role in generating functional heterogeneity among astrocytes. Support for this hypothesis has been demonstrated by LaFont et al (1992) who observed that embryonic neuron polarity can be mediated by dermatan sulfate proteoglycan, but not other proteoglycans, and that this function was dependent on the specific sugar moiety expressed rather than the protein epitope.

A variety of studies indicate that neurotrophic factors support neurite growth from central neurons (Walicke et al 1986, Hatten et al 1988, Mattson et al 1989, Kawaja and Gage, 1991). Our results indicate that in part, dendrite growth is modulated by a diffusible factor. Could the functional diversity of astroglia, therefore, be secondary to specific localization of these substances? Immunohistochemical reactivity or mRNA expression of several growth factors including bFGF (Ferrara et al 1988, Finklestein et al 1988, Gonzalez et al 1990, Gómez-Pinilli et al 1992, Logan et al 1992, Woodward et al 1992), NGF (Furukawa et al 1986, Lu et al 1991, Saad et al 1991, Yoshida and Gage 1991, Zafra et al 1992), BDNF (Zafra et al 1992), EGF (Gómez-Pinilla et al 1988, Nieto-Sampedro et al 1988), IGF (Rotwein et al 1988, Garcia-Estrada et al 1992, Chernusek 1993), PDGF (Mapstone 1991, Yeh et al 1991, Pringle et al 1992) and cytokines such as TGF (Fallon et al 1990, Wesselingh et al 1990, Saad et al 1991) and IL-1 (Wesselingh et al 1990) have been detected in astroglial cells

both *in vitro* and *in vivo*. None of these studies, however, determined whether there are regional differences in synthesis, expression or release of growth factors from astroglial cells. Recently, Lazar and Blum (1992) using a solution hybridization ribonuclease protection assay, demonstrated that the expression of EGF mRNA exhibited regional variation in the brain. It is thus conceivable that astrocytes in specific regions of the brain express or synthesize different concentrations or isoforms of growth factors.

Are there any similarities between cortex, olfactory bulb and retina that might explain their selective ability to promote dendrite growth? All are derived from the prosencephalon and are composed primarily of gray matter. Nevertheless, we observed little dendrite growth on striatal astrocytes, which are also derived from the prosencephalon and composed of gray matter. Thus, a common embryological site of origin or the relative amount of gray or white matter are unlikely explanations for the differences. One possible explanation is the interesting similarity in the basic architecture and circuitry of the three regions. The cortex, olfactory bulb and retina are all, to some extent, laminar structures composed of vertical pathways with numerous horizontal pathways that integrate the vertical signals. Thus it is conceivable that astroglia and/or neurons of these regions share common structures, surface molecules or receptors that permit astroglial mediated dendrite growth that is not entirely region specific.

Mechanism of astroglial mediated dendrite growth.

Axon and dendrite growth appear to be dissociable events and at least in part, separately regulated. Regardless of the reason for astroglial heterogeneity in promoting dendrite outgrowth, an important issue is whether dendrite

elaboration is solely dependent on molecules associated with the cell surface or regulated by soluble factors. Our findings suggest that, at least in part, astroglial-mediated dendrite growth, independent of axon growth, is promoted by a diffusible factor. This is the first direct evidence that astroglia may release a factor or factors that can selectively modulate dendritic morphology in the CNS; whether this is a novel factor or a known factor with a novel function is currently not known. Consistent with this is the recent finding by De Koninck et al (1993) that nerve growth factor (NGF) induces an increase in dendrite outgrowth, but has little effect on axon growth from neonatal rat sensory neurons.

There is considerable evidence that astroglial cells secrete diffusible neurite promoting factors, (Hatten et al 1988, Rousset et al 1988, 1990, Qian et al 1992). For example, medium conditioned on homotypic astroglia can enhance polarity of embryonic mouse mesencephalic neurons in primary culture (Rousset et al 1988 and 1990). Similarly, Seil et al (1992), have observed that medium conditioned by cerebellar astrocytes promotes the development of Purkinje cell dendritic spines. The precise molecular nature of the factor or factors responsible is not known although protein degradation studies indicate that they are proteins or require protein cofactors. While previously identified soluble peptide growth factors have been observed to promote neurite outgrowth from CNS neurons, none of these studies have reported selective effects on the growth of either axons or dendrites (Hatten et al 1988, Rousset et al 1988, Lein and Higgins 1989, Mattson et al 1989).

The simplest explanation of the data is that astroglia release a diffusible factor(s) that can specifically mediate dendrite growth independent of axon

growth. This factor could be labile or easily degraded in medium, explaining the less prolific dendrite growth observed in astroglia conditioned medium. Alternatively, close cell contact might provide a higher concentration of the factor(s). Other plausible explanations include the possibility that factors released by astroglia interact with molecules associated with the extracellular matrix or alternatively that soluble factors regulate or directly promote cell adhesion of growth cones in a process specific manner (Seilhamer et al 1987, Burgess and Maciag 1989, Rossino et al 1990, Flanagan et al 1991, Yaron et al 1991, Damsky and Werb 1992, Doherty et al 1992, Doherty and Walsh 1992, Thiery and Boyer 1992).

An important role for astroglia in modulation of dendrite growth does not exclude other mechanisms but rather emphasizes the complex integrative nature of the CNS. Further experiments will be necessary to elucidate the mechanism of astroglia mediated dendrite growth. From the present experiments it is apparent that astroglia mediate dendrite growth not only in a region specific manner, but that certain astroglia may regulate specific process outgrowth from cortical neurons independent of their anatomic origin. In part, diffusible factors may regulate astroglial mediated dendrite growth in collaboration with molecules associated with the cell surface or matrix.

**ASTROGLIA DEMONSTRATE REGIONAL DIFFERENCES IN THEIR
ABILITY TO MAINTAIN PRIMARY DENDRITIC OUTGROWTH FROM
MOUSE CORTICAL NEURONS *IN VITRO***

INTRODUCTION

Astroglia are critical for the regulation of differentiation and process outgrowth from CNS neurons (chapter 9). Neuron differentiation is accompanied by the development of two morphologically distinct processes: the axon and the dendritic arbor. There is evidence that astroglial regulation of development of axons and dendrites is differently controlled. For example, several studies have demonstrated that CNS neurons exhibit a more extensive primary dendritic arbor when cultured on astroglia derived from the same anatomic region or specialized glia such as olfactory bulb or retinal astroglia (Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Rousselet et al 1988, Prochiantz et al 1990, Rousselet et al 1990, chapter 9).

Do astroglial cells regulate the initiation or maintenance of dendrites from neurons? Several experiments suggest that glia may regulate the initiation of dendrite growth. For example, glial cells have been observed to induce dendrite initiation in sympathetic neurons (Higgins et al 1988). Sensory neurons, which do not possess dendrites *in situ*, elaborate a dendritic arbor *in vitro* when nonneuronal cells are removed and NGF is added (De Koninck et al 1993). Mesencephalic neurons demonstrate greater neuritic output (Denis-Donini et al 1984) and MAP2 expression (Chamak et al 1987) on homotypic astroglia after 2 days in culture; however, no difference is observed after longer culture periods. Similarly, Rousselet et al (1988 and 1990) found that

mesencephalic neurons extended more primary dendrites in medium conditioned by mesencephalic astroglia than in medium conditioned by striatal astroglia after only one day in culture.

Astroglial control of dendrite initiation separate from axon elaboration implies that axons and dendrites differ at the onset of process outgrowth. There is considerable evidence, however, that neurites are not differentiated at the initiation of outgrowth: 1) when neurites first emerge during development *in vitro*, axons and dendrites can not be morphologically distinguished (Dotti et al 1988); 2) if an axon is transected close to its soma soon after differentiation, a presumptive dendrite may become the axon instead (Goslin and Banker 1990, Goslin et al 1990), and 3) selective compartmentation of cytoskeletal elements (Cáceres et al 1986, Kosik and Finch 1987, Pennypacker et al 1991) or mRNA (Kleinman et al 1994) is not observed during the initial elaboration of neurites .

These data, taken together, present a paradox. If axons and dendrites do not differ in their initiation, how is it that glial cells can differ in their ability to promote the growth of these two processes at their initiation. We have previously found that astroglia demonstrate regional differences in their ability to support the number of primary dendrites elaborated by mouse cortical neurons after five days in culture (chapter 9). In this study we asked whether astroglia differ in their ability to initiate or to maintain primary dendrite growth? To address this question we used quantitative morphological and double-labeled, immunohistochemical techniques to examine neurite formation in primary cultures of embryonic mouse cortical neurons and determine: 1) the earliest time point at which regional

differences in astroglial mediated dendrite growth can be observed and 2) whether astroglia are necessary for the initiation or maintenance of the primary dendritic arbor. The data demonstrate that for embryonic mouse cortical neurons *in vitro* all the glial monolayers support the initiation of an equal number of neurites, but that astroglia derived from cortex, olfactory bulb and retina maintain a higher number of primary dendrites, whereas astroglia derived from spinal cord, mesencephalon and striatum initially support longer axons and do not maintain the primary dendritic arbor.

MATERIALS AND METHODS

Cortical neurons were prepared from E 18 mouse brains and cocultured on P4 rat astroglial monolayers derived from: cerebral cortex, striatum, mesencephalon, spinal cord, olfactory bulb and retina and on fibroblast monolayers derived from the meninges as described in chapter 7. In some experiments cortical neurons were plated on poly-D-lysine coated coverslips and grown in cortical or mesencephalic astroglial conditioned medium. Neurite outgrowth was examined at 18 hours, 1, 3, and 5 days *in vitro*. Neurites were characterized using double-labeled immunohistochemical techniques as described in chapter 8, and outgrowth quantified as described in chapter 7.

RESULTS

Changes in dendrite number during neuron development.

Previous studies have demonstrated that astroglia from different regions of the CNS differ in their ability to regulate the number of primary dendrites

that are elaborated by neurons in culture (Prochiantz et al 1990, Rousselet et al 1990, chapter 9). It is not clear, however, whether astroglia differ in their ability to initiate or maintain primary dendrite growth. Therefore, the morphologic and immunohistochemical differentiation of cortical neurons grown on various glial monolayers including those derived from cortex, retina, olfactory bulb, mesencephalon, striatum and spinal cord were investigated at 18 hours, 1, 3 and 5 days *in vitro*. The results are summarized in figure 12 and illustrated in figure 13.

Eighteen hours after plating, the majority of cortical neurons, identified by M6 or MAP2 immunoreactivity, on all glial monolayers and on fibroblasts had elaborated multiple neurites. These processes had definite features of neurites and emerged at uniform intervals around the circumference of the cell body, giving the cell a symmetric appearance. A few processes were transitional in appearance between lamellipodia and neurites. Typically, these processes were expanded and flattened proximally and exhibited more neuritic characteristics distally. Lamellipodia and transitional neurites were not included in our quantification. Neurons without processes represented less than 1% of the total population. In addition to a presumptive axon, almost all cortical neurons (90-100%) grown on each glial monolayer had four or more presumptive dendrites. Similar growth characteristics were observed on fibroblasts; 83% of neurons elaborated a presumptive axon and 4 or more presumptive dendrites. The presumptive dendrites were typically short, the majority were less than 10 μm in length. The shortest dendrites were observed on fibroblasts ($p < 0.05$). The length of presumptive dendrites on the various glial monolayers varied slightly; 20%-25% of presumptive dendrites were greater than 10 μm when neurons were grown on astroglia from

FIGURE 12: Astroglia demonstrate regional differences in their ability to maintain the primary dendritic arbor elaborated by cortical neurons in culture. Histograms illustrating axon length (a), primary dendrite number (b), primary dendrite length (c) and total dendrite output (d) from mouse cortical neurons cocultured with rat astroglia derived from different CNS regions as described in Material and Methods. Neurite elaboration was quantitatively examined in 100 randomly selected neurons growing on each glial monolayer at each time point using morphologic and double-labeling immunohistochemical techniques. Values represent the mean \pm SEM. Fibroblasts included as control for Students t-test but not for ANOVA. Whereas the number of dendrites elaborated by neurons was similar on all monolayers tested at 18 hrs *in vitro* (ANOVA $p > 0.05$; a = $p = 0.055$ vs str), astroglia derived from cortex, and retina or olfactory bulb were better able to maintain the total primary dendritic arbor than astroglia from other CNS regions (a = $p > 0.05$ vs ctx, $p < 0.05$ vs str, $p < 0.01$ vs ob; b = $p > 0.5$ vs str, $p < 0.01$ vs ctx; c = $p > 0.5$ vs mes, $p < 0.05$ vs ob; d = $p > 0.05$ vs sc, $p < 0.001$ vs ob). These regional differences were observed at 24 hrs in culture and became more pronounced with time. In particular, the maintenance of the number of primary dendrites elaborated by neurons (a = $p = 0.055$ vs str; b = $p > 0.05$ vs str, $p < 0.01$ vs ctx; c = $p > 0.5$ vs mes, $p < 0.05$ vs ob; d = $p > 0.5$ vs sc, $p < 0.01$ vs ob) and to a lesser extent, elongation of individual dendrites (a = $p > 0.05$ vs ctx, $p < 0.05$ vs str; b = $p > 0.05$ vs ctx, $p < 0.05$ vs ob, c = $p > 0.05$ vs str, ret) observed from cortical neurons was dependent on the source of astroglial cells. During early process elaboration, greater axon elongation was observed on astroglia derived from spinal cord, mesencephalon and striatum (a = $p < 0.01$ vs ctx, c = $p < 0.001$ vs ret). By 3 and 5 DIV, however, axon length was comparable on the various glial monolayers (b = $p < 0.001$ vs mes, $p > 0.05$ vs ob, ctx and d = $p > 0.05$ vs ret, ctx). (Fib = fibroblasts from meninges, SC

= spinal cord, Mes = mesencephalon, Str = striatum, OB = olfactory bulb, Ret = retina, Ctx = cortex)

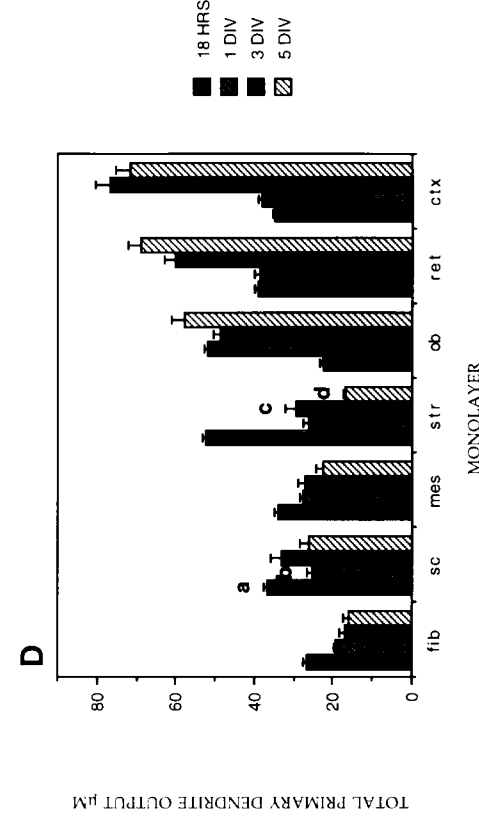
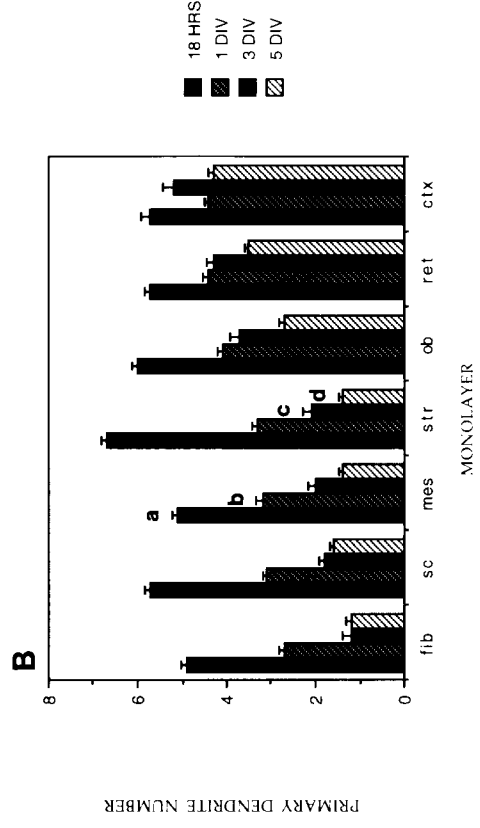
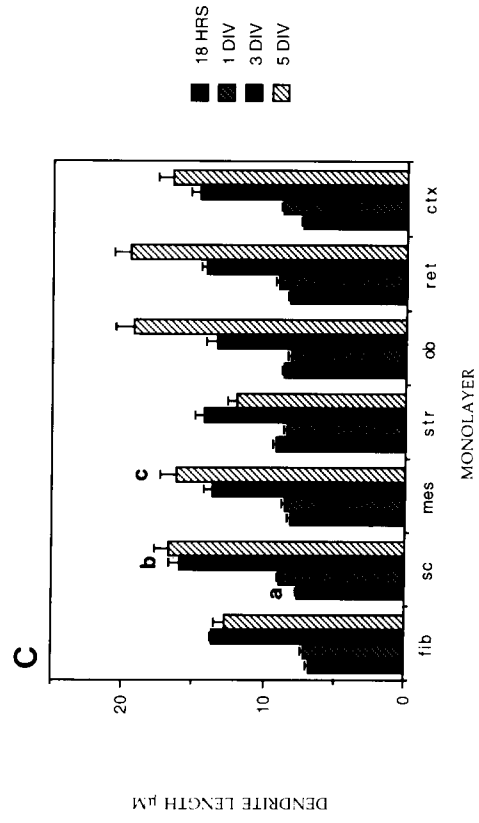
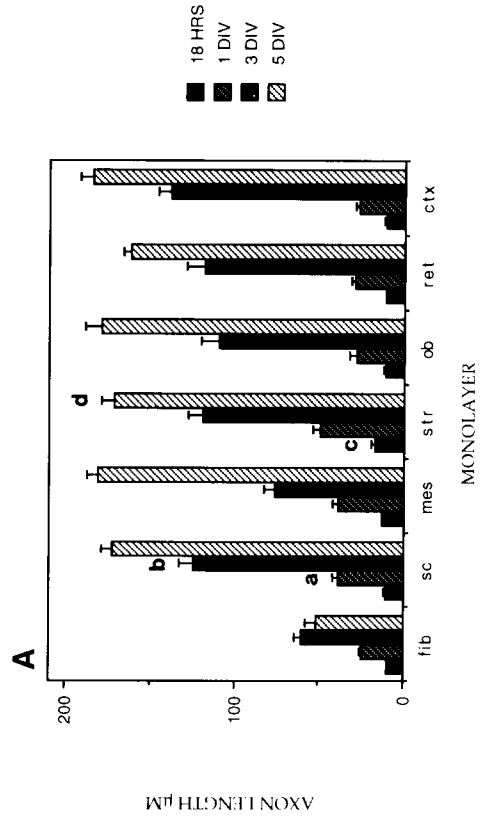
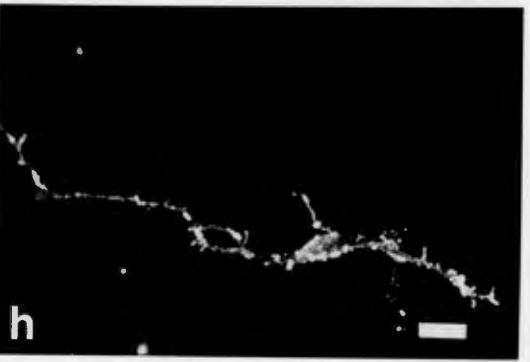
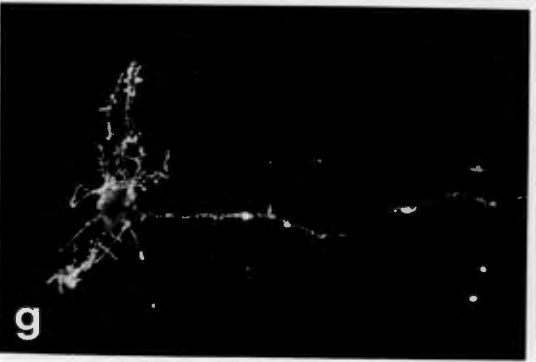
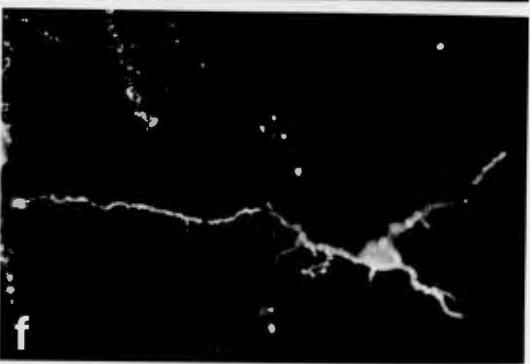
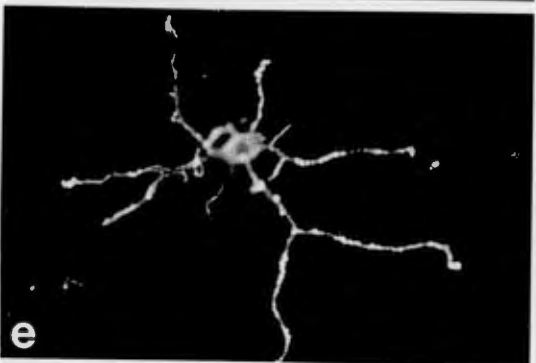
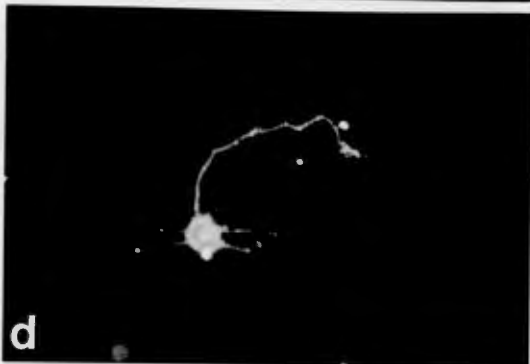
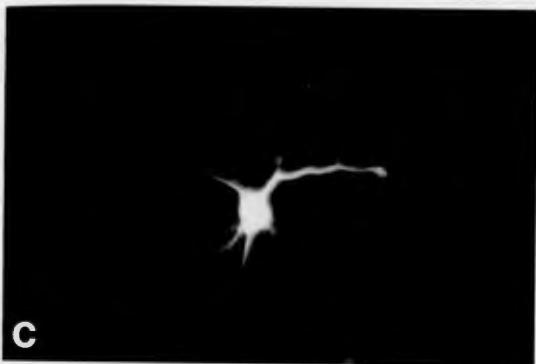


FIGURE 13: Cortical neurons growing on different glial monolayers *in vitro* exhibit dendritic differences depending on the CNS region from which the astroglia are derived. Representative fluorescent photomicrographs of E 18 mouse cortical neurons grown on cortical (a, c, e, and g) or mesencephalic astroglia (b, d, f and h) and labeled with anti-MAP2 after 18hrs (a and b) and 1 day *in vitro* (c and d), or labeled with anti-M6 after 3 (e and f) and 5 (g and h) days *in vitro*. Mesencephalic astroglia are illustrated to represent astroglia that do not maintain the primary dendritic arbor, whereas cortical astroglia are illustrated as an example of astroglia that maintain dendrite number. The majority of neurons had elaborated multiple neurites on both monolayers at 18 hours in culture. At 1 DIV neurons typically extend a long process and several shorter neurites. The number of processes, however, was different; more primary dendrites were usually observed on cortical astroglia. The morphologic difference became more pronounced after 3 days *in vitro*. Whereas axon growth was similar on monolayers tested at 5DIV; on glial monolayers derived from spinal cord, mesencephalon or striatum (h), neurons do not demonstrate an extensive dendritic arbor and usually exhibit a uni- or bipolar morphology. By contrast, on glial monolayers generated from the retina, olfactory bulb or cortex (g), most neurons are multipolar and extend an axon and several dendrites. (Scale bar = 10 μ m)



striatum, olfactorybulb and retina. By contrast only 6%-11.9% of these processes were longer than 10 μ m on spinal cord, mesencephalic or cortical astroglia.

Six hours later most neurons had one process that was significantly longer than the others. Typically the long process was 15-40 μ m longer than the next longest neurite. The total number of presumptive dendrites elaborated by the neurons on each monolayer, however, had decreased. The decline in the total number of presumptive dendrites was dependent on the type of astroglial cells on which the neurons were plated. The smallest reduction, 1.3 dendrites per cell, was observed on cortical glial monolayers. A decrease in dendrite number of similar magnitude was observed from neurons grown on olfactory bulb or retinal astroglia. A larger reduction, between 2 and 3.5 dendrites per cell, was demonstrated from neurons grown on astroglia derived from the remaining regions ($p < 0.05$). The length of individual primary dendrites, however, was similar on the different glial monolayers.

The number of primary dendrites from neurons cultured on the different glial monolayers continued to diverge with longer periods of cell culture. By 3 DIV the number of primary dendrites elaborated by neurons remained constant on retinal and cortical glial monolayers, whereas the number of primary dendrites from neurons grown on monolayers generated from spinal cord, mesencephalon and striatum continued to decline. A small, but not significant, decrease in primary dendrite number was identified on olfactory bulb astroglia. While the majority of cortical neurons cultured on olfactory bulb, retinal or cortical astroglia were multipolar (between 59.1% and 89.6% had four or more dendrites), most of the neurons cultured on astroglia

derived from spinal cord, mesencephalon or striatum had only one or two primary dendrites. With the exception of spinal cord astroglia, the length of individual primary dendrites did not differ significantly on the different glial monolayers. When neurons were grown on astroglia from the spinal cord, 48.4% of primary dendrites achieved lengths greater than 15 μ m. By contrast, between 28.2% and 37% of primary dendrites examined on other monolayers had lengths greater than 15 μ m.

The morphologic differences observed in neurons at 3 days in culture were more pronounced at 5 DIV. The majority of neurons, 54.5% to 91.6%, grown on olfactory bulb, retina and cortical astroglia elaborated three or more primary dendrites. By contrast, only 3.6% to 13.6% of neurons observed on spinal cord, striatal or mesencephalic monolayers extended three or more dendrites. Between 18 hours and 5 DIV neurons lost between 70 and 84% of their primary dendrites when cultured on monolayers derived from spinal cord, mesencephalon or striatum. In this same period, neurons grown on cortical astroglia lost only 20% of their dendrites ($p < 0.001$). An intermediate decrease, between 35% and 41%, in dendrite number was observed from neurons grown on retinal or olfactory bulb astroglia. With the exception of striatal astroglia, the length of individual primary dendrites again did not significantly differ on the different glial monolayers.

The same effects are observed when the total extent of the primary dendritic arbor is quantified. Cortical neurons cultured on glial monolayers derived from cortex, olfactory bulb and retina increased their primary dendritic arbor nearly threefold between 18 hours and 5 days in culture. By contrast, the primary dendritic arbor of neurons grown on glial monolayers from spinal

cord, striatum and mesencephalon decreased by almost half during the same time period (Figure 12).

These observations indicate that astroglia demonstrate regional differences, in their ability to maintain, but not initiate, cortical neuron primary dendrite growth. Homotypic astroglia, appear best able to maintain a primary dendritic arbor once it is elaborated. This effect is principally on dendrite number and total primary dendrite output rather than on elongation of individual primary dendrites.

Axon growth.

After 5 DIV axon length of cortical neurons did not differ significantly when plated on the various glial monolayers; 53.3%-86.6% of axons examined were greater than 150 μ m in length. By contrast, all axons on fibroblast monolayers were less than 100 μ m in length. There was a tendency for greater axon lengths from cells cultured on cortical astroglia; however the difference was not statistically significant, ($p>0.05$; Figure 12). Since the mouse neurons were plated at low density, the entire length of individual axons could be followed and quantified.

During early process outgrowth the extent of axonal elongation differed on the various glial monolayers. With the exception of striatal astroglia, axon length was similar on the various monolayers at 18 hours *in vitro*. In the 6 hour period between 18 hours and 24 hours *in vitro*, however, a three to fourfold increase in mean axon length was observed from cortical neurons grown on spinal cord, mesencephalic and striatal astroglia whereas axon length from cortical neurons only increased two to threefold on the

remaining cell monolayers, including fibroblasts. Thus at 1 DIV average axon length was between 38.4 μ m and 49.6 μ m on spinal cord, mesencephalic and striatal astroglia, whereas average length of axons was between 24.5 μ m and 29.4 μ m on fibroblasts or cortical, retinal or olfactory bulb astroglia (Fig 12).

The early difference in astroglial support of axon growth is also reflected in the difference in length between the longest and second longest neurite elaborated from each neuron on monolayers derived from different CNS regions (Table 6). As observed above, at 18 hours, there were no significant differences between the longest and second longest neurites in most cortical neurons regardless of the source of the glial monolayers. Furthermore this difference, with the exception of striatal astroglia, was similar on the various monolayers. In the next six hours, however, the mean difference in lengths between the two longest neurites was twofold greater from neurons grown on spinal cord, mesencephalon and striatum derived astroglia than those observed on cortex, olfactory bulb or retinal astroglia (Table 6, $p < 0.05$). These data demonstrate that astroglia derived from spinal cord, mesencephalon and striatum support greater initial axon elongation than observed from neurons grown on olfactory bulb, retinal or cortical astroglia. This support of axonal elongation corresponds to the period of time when the total number of processes begins to decline in these cells. At subsequent time periods (3 and 5 DIV), however, astroglial support of axon growth fails to demonstrate any correlation with a particular group of astroglia.

Astroglia are necessary to maintain the primary dendritic arbor.

To verify that astroglia maintain rather than initiate primary dendrite growth from cortical neurons *in vitro* we performed the following experiment. In a

TABLE 6: THE TIME COURSE OF AXON ELONGATION DIFFERS ON VARIOUS ASTROGLIAL MONOLAYERS.

| Monolayer [¶] | 18 Hours | 1 DIV | 3 DIV | 5 DIV |
|-------------------------|------------|-------------|--------------|---------------|
| Fibroblast [§] | 1.65±0.16 | 15.74±0.81 | 46.94±2.8* | 30.69±1.93* |
| Spinal cord | 2.28±0.21 | 27.68±1.35* | 107.63±4.44 | 155.76±2.8 |
| Mesencephalon | 3.1±0.25 | 27.5±1.74 * | 64.86±3.01* | 168.62±3.01 |
| Striatum | 4.55±0.03* | 39.76±1.83* | 106.88±3.83 | 158.44±3.61 |
| Olfactory bulb | 1.27±0.05 | 17.87±1.41 | 89.39±5.09 † | 158.64±3.27 |
| Retina | 1.11±0.08 | 17.74±0.76 | 99.39±5.94 | 140.32±2.04 * |
| Cortex | 1.83±0.12 | 17.59±0.94 | 116.18±5.3 | 165.33±3.16 |

Table 6: Difference between longest and second longest process, in μm , elaborated by mouse cortical neurons on various cell monolayers. During early process outgrowth the extent of axon elongation differed on the various glial monolayers. Glial monolayers derived from spinal cord, mesencephalon or striatum supported longer axons at 1DIV than neurons grown on astroglia derived from olfactory bulb, retina or cortex. The different abilities of astroglia to support axon growth was confined to this early phase of culture only, as axon length and the difference in length between the longest and second longest process did not demonstrate a correlation with a particular group of astroglia at subsequent time periods. Values represent the mean \pm SEM; n=100 neurons for each astroglial subtype at each time point. ([¶] CNS region from which the glial monolayer is derived; [§] fibroblasts, derived from the meninges, represent controls; * = $p < 0.05$; † = $p = 0.051$ compared with cortex at the same time in culture).

previous study (see chapter 9) we found that soluble factors are, at least partly, responsible for the different capacities of astroglia to support dendrite number. Therefore in the present study, we plated E18 mouse cortical neurons at low density (1×10^4 cells/ml) directly onto polylysine coverslips for 24 hours in conditioned medium from either mesencephalic astroglia or cortical astroglia, and after 24 hours *in vitro* exchanged the media so that cortical neurons initially exposed to cortical astroglia were now grown in medium conditioned by mesencephalic astroglia (CM) whereas cortical neurons first exposed to mesencephalic astroglia were now exposed to cortical astroglia (MC). Neurite outgrowth was analyzed after 5 days *in vitro*. The results of this analysis are illustrated in table 7.

The results are consistent with the conclusion that astroglial cells differ in their ability to support, but not initiate dendrite growth. In medium conditioned by cortical astroglia for the first 24 hours *in vitro* and mesencephalic astroglia thereafter, only 18.1% of neurons examined had 3 or more dendrites, whereas 49% of neurons grown in medium conditioned by mesencephalic astroglia for the first 24 hours *in vitro*, and cortical astroglia thereafter had three or more dendrites ($p < 0.01$; Table 7). The length of individual primary dendrites from cortical neurons did not differ significantly between the different glial monolayers ($p = 0.078$). These observations indicate that astroglial cells release factors into the culture medium which can specifically maintain the number of primary dendrites elaborated by cortical neurons. By contrast, these factors released by astroglia are probably not responsible for the initiation of primary dendrite growth.

TABLE 7: REVERSION EXPERIMENTS

| | Axon length, μm | Dendrite length, μm | Number of primary dendrites | Total primary dendrite output, μm |
|---|----------------------------|--------------------------------|--------------------------------|---|
| Mesencephalic astroglia, 1DIV | 38.39 ± 1.71 | 8.62 ± 0.24 | 3.19 ± 0.13 a | 27.5 ± 0.91 |
| Mesencephalic astroglia, 5DIV | 181.4 ± 3.06 | 16.14 ± 0.18 | 1.4 ± 0.06 | 22.59 ± 1.67 |
| Cortical astroglia, 1DIV | 26.853 ± 0.96 | 8.79 ± 0.17 | 4.41 ± 0.11 b | 37.89 ± 1.03 |
| Cortical astroglia, 5DIV | 184.7 ± 2.97 | 16.62 ± 1.06 | 4.3 ± 0.12 | 71.66 ± 3.44 |
| CM, Cortical then mesencephalic astroglia | 184.42 ± 5.03 c | 18.65 ± 0.97 | 1.36 ± 0.11 | 25.43 ± 1.09 |
| MC, Mesencephalic then cortical astroglia | 247.1 ± 1.09 | 13.33 ± 0.41 | 2.67 ± 0.08 d | 38.12 ± 0.64 |

Table 7: Astroglia maintain, rather than initiate cortical neuron dendrite growth. Mouse cortical neurons were grown in a noncontact coculture system in which the medium was continuously conditioned by astroglia derived from the mesencephalon or cortex for 24 hours. Neurons were then grown in the opposite conditioned medium. Axon length, primary dendrite number, dendritic length and total primary dendritic output was quantified from 100 randomly selected neurons from three experiments for each astroglial subtype after 5 DIV. Results represent the mean \pm SEM. Values for neurons grown on either mesencephalon or cortical astroglia at 1 and 5DIV are illustrated for comparison. (a) $p > 0.05$ compared with MC; b) $p = 0.001$ compared with CM; c) $p < 0.05$ compared with MC; d) $p < 0.01$ compared with CM.)

DISCUSSION

Previous work has demonstrated that astroglial cells derived from different regions of the CNS are heterogeneous in their abilities to support dendrite growth from mouse cortical neurons *in vitro* (Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Rousselet et al 1988, Prochiantz et al 1990, Rousselet et al 1990, chapter 9). The results from the experiments described in this chapter demonstrate that this difference is due to differential maintenance of primary dendrite number, rather than differences in the ability of various astroglia to induce the initiation of dendritic processes. The cortical neurons initially elaborate the same number of neurites when cultured on glial monolayers, regardless of the region from which the cells were derived. Surprisingly, even fibroblast monolayers initially supported the same number of neurites from cortical neurons. Within 24 hours, however, the astroglial cells began to demonstrate differences in their ability to maintain dendritic growth, and these differences became progressively more pronounced with continued culture.

The results of this study further suggest that the number of primary dendrites maintained by cortical neurons *in vitro* may be associated with the cell's initial axon growth. The decline in neurite number from cortical neurons grown on spinal cord, mesencephalic or striatal glial monolayers is first discernible at the same time that the axonal process can be distinguished from the other neurites. In addition to this temporal correlation, we have found that those astroglial cells that initially support longer axons are the same as those that subsequently sustain the fewest dendrites. These results suggest that astroglial support of axon growth may occur at the expense of dendrite

growth; however, this effect appears only to be confined to the early phase of axonal elongation since axonal growth is subsequently comparable regardless of the source of the glial monolayer. Interestingly, the effect appears to be exerted primarily on dendritic number, rather than on dendritic length. These findings are consistent with previous reports that have observed an inverse relationship between the extent of axon and dendrite growth in developing neurons in culture (Kosik and Finch 1987, Chamak and Prochiantz 1989, Cáceres et al 1991, Lochter and Schachner 1993). For example, Chamak and Prochiantz (1989) observed that accelerated axon growth form mesencephalic neurons cultured with either substrate bound fibronectin or soluble laminin was associated with an almost complete absence of dendritic growth.

As noted in the Introduction of this chapter, previous studies have provided evidence both for glial mediated initiation of dendritic growth, and also heterogeneity in the ability of glial cells to promote different numbers of dendrites from neurons, depending on the region of the CNS from which the neurons were derived. The results of this study suggest that the two phenomena may not be directly related. All of the glial monolayers supported neurite initiation to the same degree, consistent with previous observations that glia can mediate dendrite initiation. For example, when glia are added to sympathetic neurons in culture, the neurons promptly initiate a dendritic arbor (Higgins et al 1988). We have found that the differences between astroglia in their support of dendrites is detectable only after 24 hours, when neurons cultured on certain glial monolayers begin to lose their primary dendrites. Since all other studies that have reported glial heterogeneity in this property have assayed the cells after 24 hours or longer *in vitro*, our results

would suggest that these previous studies were also assessing differing abilities in the maintenance of dendrite number, rather than initiation of dendrites (Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Rousset et al 1988, Tropea et al 1988, Johnson et al 1989, chapter 9).

Further support for the possibility that astroglia demonstrate regional difference in their ability to maintain dendrite number comes from the results of the conditioned medium experiments described in this chapter. When neurons were cultured in medium conditioned by mesencephalic astroglia, a source of astroglia that maintains dendrite number poorly, for the first 24 hours and for the subsequent 4 days in medium conditioned by cortical astroglia, the number of dendrites observed was similar to that of neurons grown on mesencephalic astroglia alone for 24 hours. This result indicates that cortical astroglia were unable to initiate further dendrite outgrowth.

Taken together our results suggest that dendritic development involves an initial stage of proliferation followed by retraction; branches that are initially present may disappear altogether. The degree of retraction is determined by the local cellular environment. Ramon y Cajal was the first to observe that the young neuron undergoes a period of tremendous proliferation and profusion of dendritic processes many of which are eliminated in later life. This phenomenon has been verified by many investigators in various parts of the nervous system both *in vivo* and *in vitro* (Weiss and Pysh 1978, Smith 1981, Rogowski and Feng 1982, Purves et al 1986, Role and Fischbach 1987, Dotti et al 1988, Purves et al 1988, Ramoa et al 1988, Sanes et al 1992a and 1992b).

Many factors in the local microenvironment have been demonstrated to be important regulators of dendritic remodeling (Van der Loos 1965, Rubel et al 1981, Young and Poo 1983, Deitch and Rubel 1984, Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Rousselet et al 1988, Mattson et al 1988a, Chamak and Prochiantz 1989, Lipton and Kater 1989, Mattson et al 1989, Prochiantz et al 1990, Rousselet et al 1990, Schilling et al 1991, Sanes et al 1992a and 1992b). Our results indicate that astroglial cells may be important in controlling the size of the primary dendritic arborization. Qualitative and quantitative *in situ* Golgi analysis of the rodent cortex (Wise et al 1979, Petit et al 1988) or brainstem (Hammer et al 1981) demonstrate that an immature dendritic arbor composed almost entirely of primary dendrites is formed early in development and is present before the arrival of afferents and establishment of synapses. Subsequent dendritic development occurs postnatally after the arrival of axons and is observed principally in the distal dendritic tree, i.e. non terminal secondary or tertiary branches. Furthermore, the number and length of primary dendrites remains constant once branching has occurred (Petit et al 1988). Developmental studies in other parts of the nervous system similarly demonstrate that the primary dendritic arbor is present early in development prior to the arrival of afferents and largely independent of the presence of preganglionic innervation (Voyvodic 1987, Snider et al 1992, Sutton et al 1992). The basic form of the neuron, its primary neuritic arbor is therefore established before neuronal input. Our results suggest that the local astroglial environment maintains the primary neuritic arbor; prior to the arrival of afferents this may be critical for establishing appropriate and specific connections in the CNS. Once appropriate synapses

are established, neuronal activity may selectively refine dendritic morphology by promoting or removing secondary and tertiary dendrites.

**REACTIVE AND MATURE ASTROGLIA SUPPORT PRIMARY DENDRITIC
BUT NOT AXONAL OUTGROWTH FROM MOUSE CORTICAL NEURONS
IN VITRO.**

INTRODUCTION

A striking characteristic of astrocytes is their vigorous response to a variety of CNS insults such as trauma or ischemia. The response to injury, known as reactive astrogliosis is distinguished by hyperplasia, hypertrophy, extension of processes and an increase in glial fibrillary acidic protein (GFAP) expression in astrocytes (Dahl and Bignami 1976, Berry et al 1983, Mathewson and Berry 1985, Topp et al 1989, Balasingam et al 1994). The characteristics of reactive astrocytes found *in vivo* are likewise demonstrated by injured astroglia in culture (Rudge and Silver 1990, McKeon et al 1991, Shepard et al 1991, Yu et al 1993, McMillan et al 1994). These dramatic changes are thought to have a variety of functional consequences which may be both beneficial and detrimental to the injured CNS.

Several lines of evidence suggest that reactive astrocytes do not support neurite growth and may even be impediments to axon regrowth (Reier et al 1983, Liuzzi and Lazek 1987). First, ultrastructural analysis of regrowing axons demonstrates growth arrest in the immediate vicinity of reactive astrocytes (Reier et al 1983, Liuzzi and Lazek 1987, Reier et al 1992). Second, reactive astrocytes *in vivo* can express surface molecules that limit neurite extension *in vitro* (McKeon et al 1991, Laywell et al 1992). Third, explants of injured rat cortex (Rudge and Silver 1990) and astrocyte cell lines that express cell surface

characteristics of reactive astroglia *in vivo* (Groves et al 1993), are less effective than neonatal astrocytes at promoting neurite outgrowth *in vitro*.

Following injury, the mature mammalian CNS demonstrates vigorous reactive astrogliosis and rarely supports axon regrowth. By contrast, the immature CNS demonstrates mild reactive astrogliosis and can support axon regrowth (Berry et al 1983, Barrett et al 1984, Moore et al 1987, Trimmer and Wunderlich 1990, Balasingam et al 1994). Observations *in vivo* and *in vitro* demonstrate that as the normal astrocyte matures the ability to support neurite growth decreases (Smith et al 1986 and 1990); reduced reactive astroglial support of neurite growth may, therefore, represent a functional difference between mature and immature astroglia.

Dendrite growth and remodeling, in contrast to axon growth, have been observed to continue in the adult nervous system (Purves et al 1986, Coleman and Flood 1986) and following injury dendritic proliferation and hypertrophy is thought to play a role in recovery of neuronal function (Coleman and Flood 1986, Jones and Schallert 1994). There is compelling evidence that axon and dendrite growth is differently regulated by normal astrocytes (Denis-Donini et al 1984, Chamak et al 1987, Rousset et al 1988 and 1990, chapter 9); these data raise the possibility that the differences observed in axon and dendrite growth after injury, or in the mature CNS may, in part, be due to differences in the ability of reactive or mature astroglia to support the growth of these distinct types of processes. To test this hypothesis we quantified neurite growth from embryonic mouse cortical neurons cultured on normal, mature, or reactive glial monolayers using quantitative morphological and double-labeling immunohistochemical techniques. The data demonstrate

that for embryonic mouse cortical neurons *in vitro* mature and both neonatal or mature reactive astroglia exhibit a reduced ability to support axon growth. By contrast, mature and reactive astroglia are able to support the primary dendritic arbor.

MATERIALS AND METHODS

Dissociated E18 mouse cortical neurons were cultured at low density on: 1) normal astroglial monolayers derived from neonatal (P4) rat cerebral cortex and mesencephalon, 2) mature astroglial monolayers (Smith et al 1990) derived from late postnatal (P12) rat cerebral cortex and mesencephalon, and 3) monolayers of reactive astroglia derived from P4 and P12 lesioned rat cortex. Details are provided in chapter 7. After 5 days *in vitro*, axon and dendrite growth from isolated cortical neurons was quantified using double-labeled immunohistochemical techniques as described in chapter 7. Three experiments were performed for each condition. Approximately 100 neurons were examined on each glial monolayer.

RESULTS

Characterization of cellular monolayers.

Astroglial maturation and injury alter their ability to support neurite growth *in vitro* (Smith et al 1990, Rudge and Silver 1990, McKeon et al 1991). Astroglia derived from different CNS regions also differ in their ability to support neurite growth *in vitro* (Denis-Donini et al 1984, Chamak et al 1987, Rousselet et al 1988 and 1990, chapter 9). In the present study, therefore, mesencephalic and cortical astroglia were derived from P4 (immature) or P12

(mature) rats and reactive cortical astroglial cells were derived from P4 and P12 rats 3 days following penetrating cortical injury. Representative cultures were labeled with a nuclear stain and cell type specific antibodies; the immunohistochemical and morphological characteristics of the various monolayers are illustrated in Table 8.

The major cell type occupying all glial cultures were astroglia or reactive astroglia. A slight increase in the percentage of fibroblasts was observed in cultures obtained from older or injured animals (Table 8). The majority of cells in immature (P4) and mature (P12) cultures derived from mesencephalon and cortex demonstrated morphologic and immunologic characteristics of type 1 astrocytes *in vitro* (Table 8). Qualitatively, P12 astroglia demonstrated stronger GFAP immunolabeling. In general, the immature cultures appeared more heterogeneous.

The majority of cells obtained from lesioned cortex were reactive astroglia (Table 8) and extended swollen, elongated cytoplasmic processes that expressed GFAP. Hypertrophy and lobulation of the nuclei were observed in some cells. Reactive astroglia were larger, extended more processes and qualitatively were more strongly labeled with GFAP than normal astroglia. Vimentin coexpression with GFAP and rare small GFAP negative cells were occasionally observed. Reactive astroglia derived from older lesioned animals demonstrated greater GFAP immunoreactivity, elaborated more processes and formed a more haphazard monolayer when compared with cells obtained from lesioned P4 cortex.

TABLE 8: IMMUNOHISTOCHEMICAL CHARACTERISTICS OF CELLULAR MONOLAYERS

| Cell type | Antigenic marker | MEN | P4 MES | P12 MES | P4 CTX | P12 CTX | RX P4 | RX P12 |
|--------------------|------------------|------|--------|---------|--------|---------|-------|--------|
| Type I astrocyte | GFAP+/A2B5- | - | 83.5 | 85 | 89.5 | 88.6 | - | - |
| Reactive astrocyte | GFAP+/A2B5- | - | - | - | - | - | 87.7 | 86.5 |
| Type II astrocyte | GFAP+/A2B5+ | - | 2 | - | 3.7 | 1.2 | - | - |
| O2A progenitor | GFAP-/A2B5+ | - | - | - | - | - | - | - |
| Oligodendrocyte | GFAP-/GAL-C+ | - | 1.5 | - | 2.3 | 2 | - | 1 |
| Fibroblast | Thy 1.1 + | 92.6 | 4.2 | 6.5 | 2.2 | 7.1 | 5.7 | 12.5 |
| Neuron | MAP2+/NF+ | - | - | - | - | - | - | - |
| Immature glia | VM+ | - | 53 | 10 | 83.5 | 42 | 10 | 5 |
| Microglia | OX42+ | - | 1.2 | 1.9 | - | 1.1 | 2.3 | - |
| Mouse contaminant | M2+/M6+ | - | - | - | - | - | - | - |

Table 8: Astroglia were derived from P4 and P12 rat cortex or mesencephalon and reactive astroglia from P4 and P12 lesioned rat cortex as described in Materials and Methods and characterized with cell specific antibodies. Data are expressed as the percentage of total cells identified by a nuclear stain (DAPI) that are immunoreactive for a specific antibody (- indicates not detected). Five random fields from 5 coverslips of each monolayer were examined from 3 independent cultures. Immunohistochemical characterization demonstrates: 1) the complete absence of neurons and 2) the majority of cells are GFAP positive and therefore represent astroglia or reactive astroglia. Reactive astroglia were larger, extended longer and more processes and demonstrated more prominent nuclei than normal astroglia (Men = meninges, Mes = mesencephalon, Ctx = cortex, RX = injured cortex)

Dendrite growth is supported by immature and mature astroglia.

Astroglial age did not influence the extent of dendrite growth (Figure 14 and 15). The majority of neurons, 91.6% and 94.4%, grown on P4 or P12 purified cortical glial monolayers, respectively, elaborated three or more primary dendrites. The length of individual primary dendrites did not differ significantly, although there was a tendency for longer primary dendrites to be observed on P12 cortical astroglia. Total primary dendritic elaboration exceeded 50 μ m from 82.5% and 60% of neurons grown on monolayers derived from P4 and P12 cortex respectively.

Cortical neurons grown on monolayers derived from the mesencephalon did not demonstrate an extensive dendritic arbor; astroglial age, however, did not influence the extent of the arbor (Figure 14 and 15). Only 6.6% of neurons observed on P4 and no neurons grown on P12 mesencephalic astroglia extended three or more MAP2 immunoreactive processes. Mean total primary dendritic outgrowth was approximately threefold greater on astroglia derived from P4 or P12 cortex than that observed on astroglia derived from P4 ($p<0.001$) or P12 ($p<0.001$) mesencephalon. Immature and mature mesencephalic astroglia, however, achieved similar total primary dendritic arbors; 8% and 0% of neurons examined on P4 mesencephalic and on P12 mesencephalic glial monolayers respectively extended a total primary dendritic arbors greater than 50 μ m (Figure 15).

Mature astroglia exhibit a reduced capacity to support axon growth.

Mature astroglia derived from P12 cortex or mesencephalon demonstrated a reduced capacity to support axon elongation (Figure 14 and 15); axon length, however, was not influenced by the anatomic source of the glial monolayer.

FIGURE 14: Mature astroglia are able to support dendrite growth, but not axon growth from cortical neurons *in vitro*. Representative fluorescent photomicrographs of E18 mouse cortical neurons grown on astroglial monolayers derived from P4 cortex (a), P12 cortex (b), P4 mesencephalon (c), and P12 mesencephalon (d), and labeled with anti-M6 after 5 days *in vitro*. Whereas similar dendrite output is observed from cortical neurons (arrows) grown on monolayers derived from P4 (a) or P12 (b) cerebral cortex, axon length (arrowhead) is reduced on P12 astroglia (d). On monolayers derived from mesencephalon (c and d), neurons do not demonstrate an extensive dendritic arbor (arrows); astroglial age, however, does not influence the extent of the arbor. By contrast, P12 mesencephalic astroglia (d) demonstrate a reduced ability to support axon growth (arrowhead). (Scale bar = 10 μm)

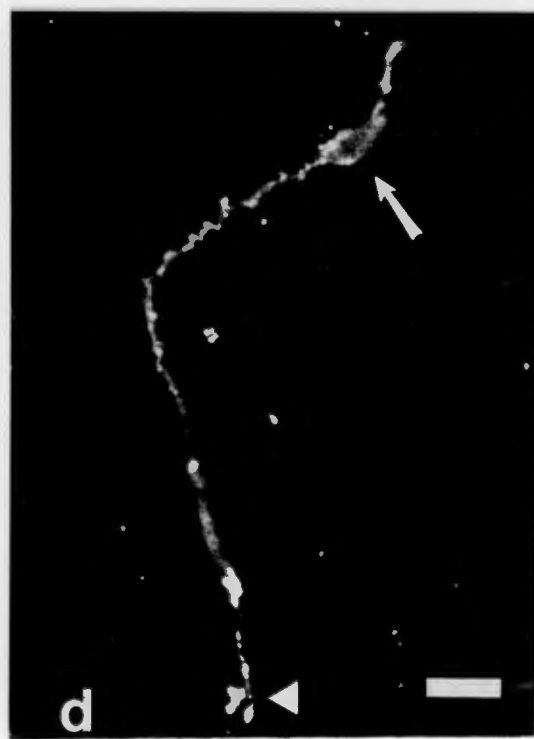
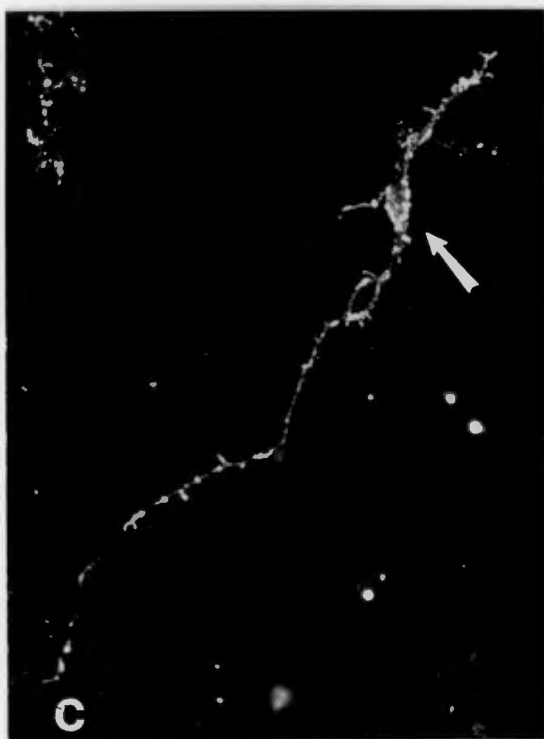
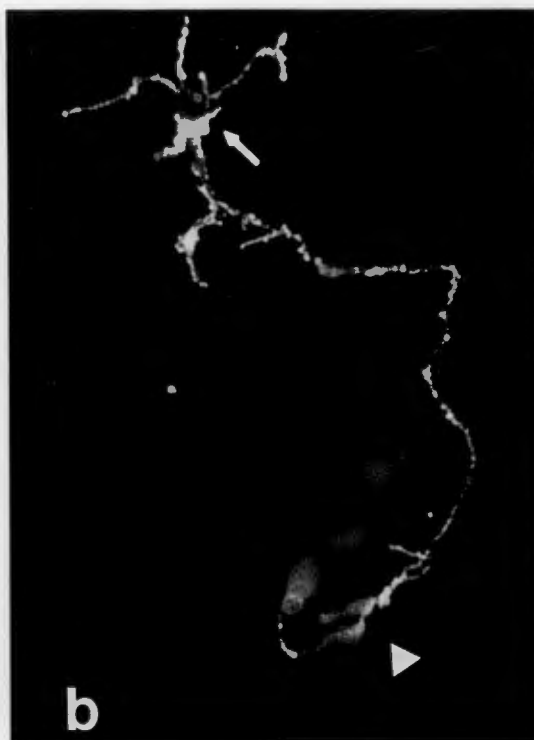
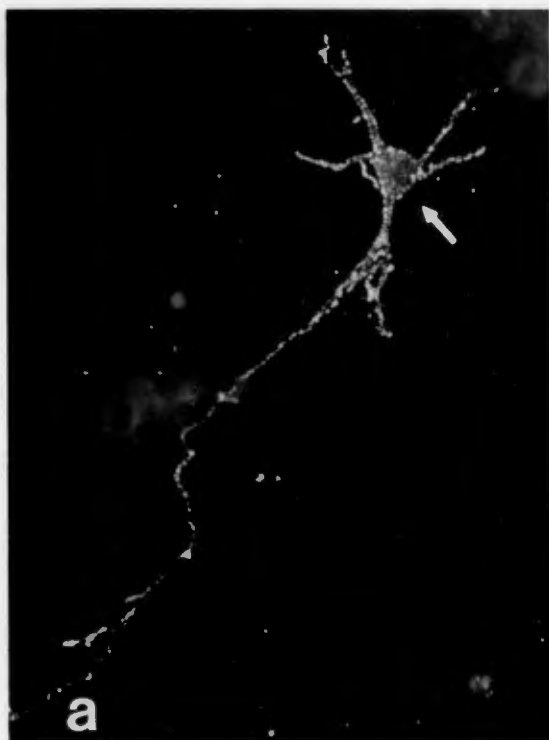
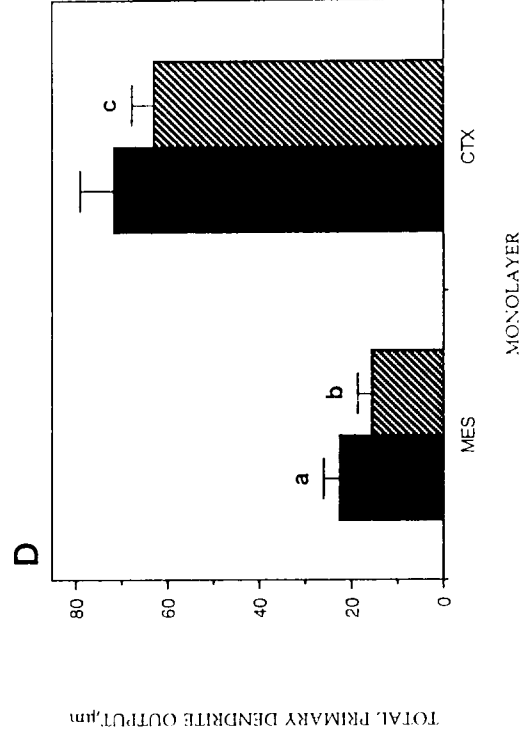
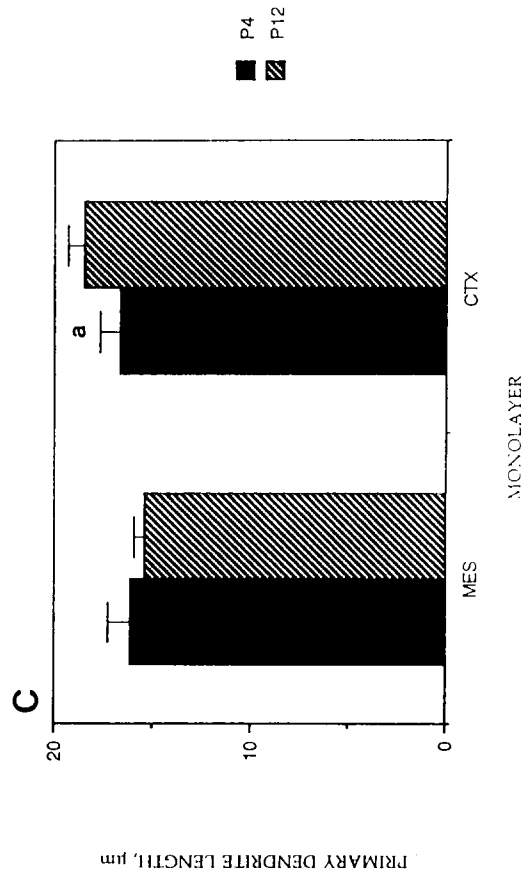
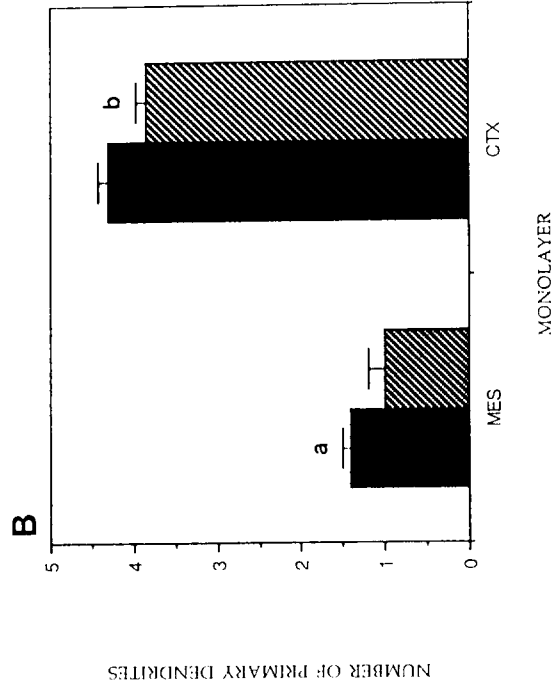
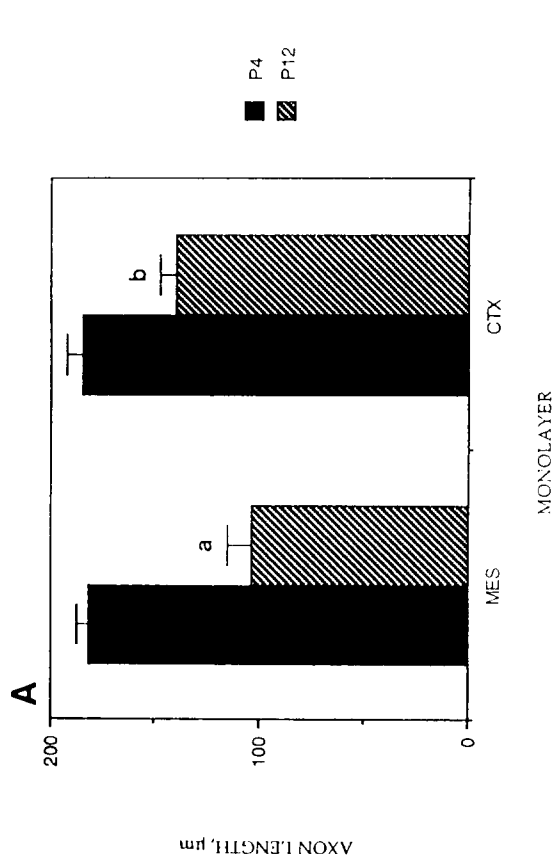


FIGURE 15: Dendrite growth reflects the region of the CNS from which the astroglia are derived and not the age of the animal. By contrast, mature astroglia demonstrate a reduced ability to support axon growth from mouse cortical neurons *in vitro*. Histograms illustrating axon length (a), primary dendrite number (b), primary dendrite length (c) and total dendrite output (d) from mouse cortical neurons cocultured with rat astroglia derived from P4 and P12 cortex and mesencephalon as described in Materials and Methods. Neurite elaboration was examined in 100 randomly selected neurons growing on each glial monolayer using quantitative analysis and double-labeling immunohistochemical techniques. Values represent the mean \pm SEM. Axon length was decreased on both cortical and mesencephalic P12 glial monolayers (a = $p < 0.001$ vs P4 mes; b = $p < 0.001$ vs P4 ctx and $p < 0.05$ vs P12 mes). Total output of primary dendrites was threefold greater on astroglia derived from P4 or P12 cortex than for astroglia from P4 or P12 mesencephalon (a = $p < 0.001$ vs P4 ctx; b = $p < 0.001$ vs P12 ctx). Astroglia derived from the same anatomic region did not demonstrate an age related difference in supporting total dendrite output (b = $p > 0.1$ vs P4 mes and c = $p > 0.1$ vs P4 ctx). An increase in the number of primary dendrites (a = $p < 0.001$ vs P4 ctx; b = $p > 0.1$ vs P4 ctx) and to a lesser extent, elongation of individual dendrites (a = $p > 0.1$ vs P12 ctx and P4 mes) observed from cortical neurons was dependent on the source, but not age of astroglial cells. (Mes = mesencephalon, Ctx = cortex)



Since the mouse neurons were plated at low density, the entire length of individual axons could be followed and quantified. Axon length was decreased when the neurons were grown on mature (P12) cortical astroglia ($p < 0.001$); 39.2% of neurons extended axons greater than 150 μm in length, whereas 93.8% of axons examined were greater than 150 μm in length when cortical neurons were plated on P4 cortical monolayers. Similar results were observed on mesencephalic glial monolayers; 14.2% of axons examined were greater than 150 μm in length when grown on P12 mesencephalon whereas 86.2% of cortical neurons extended axons greater than 150 μm in length when grown on astroglia derived from P4 mesencephalon ($p < 0.001$).

These immunohistochemical observations demonstrate that astroglia exhibit regional anatomic, but not age related differences in their ability to support primary dendrite growth from mouse cortical neurons *in vitro*. By contrast, whereas axon growth was similar on monolayers derived from different CNS regions, mature astroglia demonstrated a reduced ability to support axon growth.

Reactive cortical astroglia demonstrate a reduced ability to support axon growth but not dendrite number.

A significant morphologic difference was observed when neurite output from cortical neurons grown on reactive astroglia was compared with neurite growth on normal astroglia derived from animals of the same age (Figure 16). Cortical neuron axon length was reduced when plated on P4 reactive astroglia ($p < 0.001$); 71.5% of axons were less than 150 μm in length whereas on non reactive astroglia, 93.8% of axons achieved lengths greater than 150 μm . In contrast to axon growth, primary dendrite number, primary dendrite length

and total primary dendritic output from neurons grown on astroglia derived from P4 lesioned or normal cortex were similar (Figure 16 and 17).

To determine whether a more vigorous reactive astroglial response was associated with a greater attenuation of neurite elaboration, axon and dendrite growth from cortical neurons were quantified on astroglia derived from lesioned P12 rat cortex (Figure 17). On P12 reactive astroglia there was a significant decrease in axon growth, all axons were less than 150 μ m in length. The relative reduction of axon length on P4 reactive or P12 reactive astroglia compared to normal astrocytes of the same age, however, was not different ($p>0.05$). The length of individual primary dendrites elaborated by neurons on P4 or P12 reactive astroglia was not significantly different ($p>0.05$); however, the mean reduction in length compared with normal astroglia of the same age was greater on P12 reactive astroglia ($p<0.001$). The number of primary dendrites and total dendrite output observed on P12 reactive astroglia was similar to that observed from neurons grown on normal P4 and P12 astroglia.

Reactive astroglia may release diffusible factors that support neurite growth.

Previous investigations have demonstrated that normal astroglial cells secrete diffusible neurite promoting factors (Hatten et al 1988, Rousselet et al 1988 and 1990, Qian et al 1992, chapter 9). To determine whether mature, reactive astroglia also release diffusible neurite promoting factors, we compared the number and length of neurites from cortical neurons grown on glial monolayers with those grown in a noncontact coculture system in which the astroglia continuously conditioned the culture medium.

FIGURE 16: Reactive astroglia are able to support dendrite growth from cortical neurons *in vitro*. Representative fluorescent photomicrographs of: 1) cell monolayers derived from P4 cortex (a) and P4 cortex 3 days following a penetrating injury (c) labeled with anti-GFAP (a and c) and 2) E18 mouse cortical neurons labeled with anti-M6 after 5 days *in vitro* (b, d). The majority of cells from non-lesioned animals demonstrated a flat polyglonal morphology and could be classified as astrocytes. By contrast, cells derived from lesioned cortex demonstrated multiple processes and could be classified as reactive astrocytes. On glial monolayers generated from the nonlesioned and lesioned P4 cortex, most neurons are multipolar and extend an axon and several dendrites (b, d). Whereas dendrite output is similar on normal and reactive astroglia, axon length (arrowhead) is reduced on reactive astroglia (d). Neurons grown on normal or reactive astroglia derived from P12 animals exhibit similar morphology to that observed on P4 astroglia. (Scale bar = 10 μm)

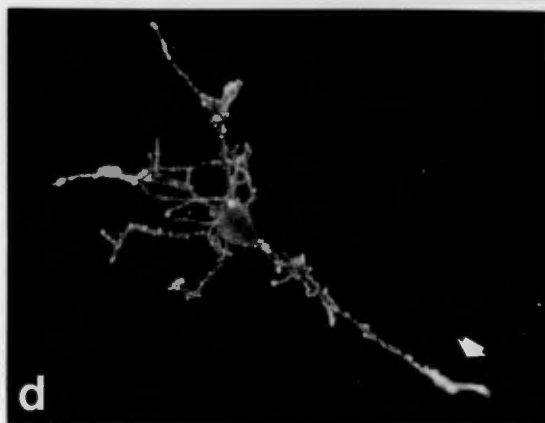
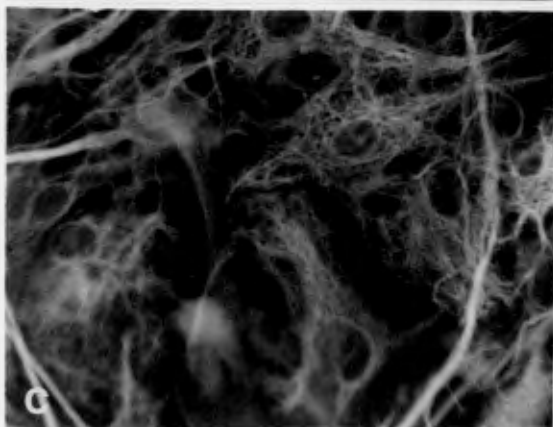
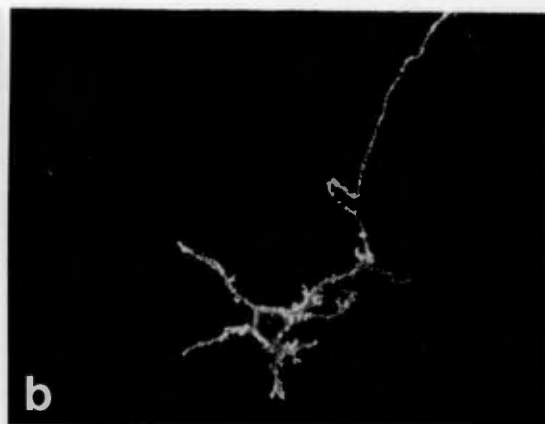
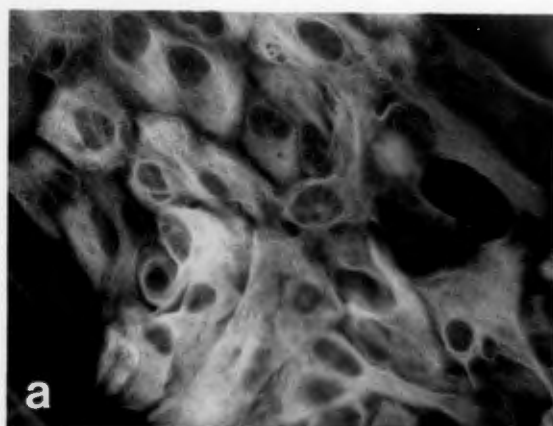
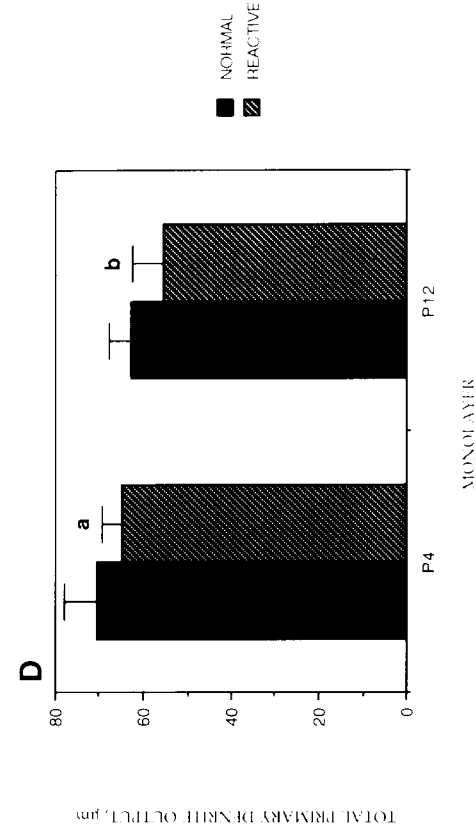
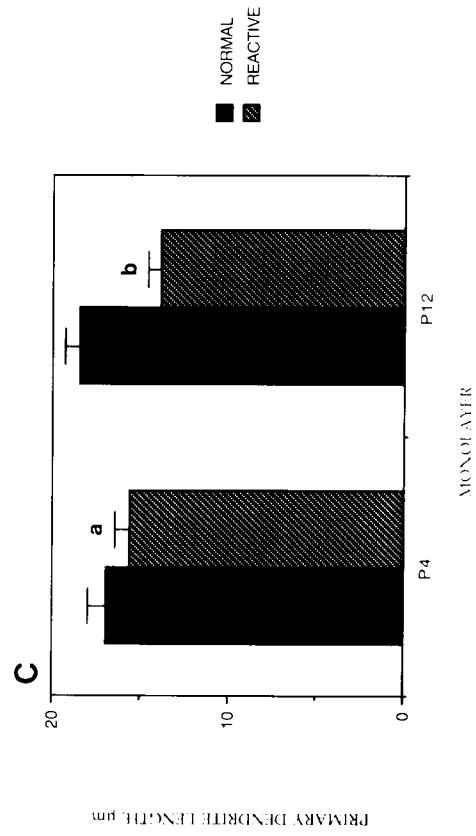
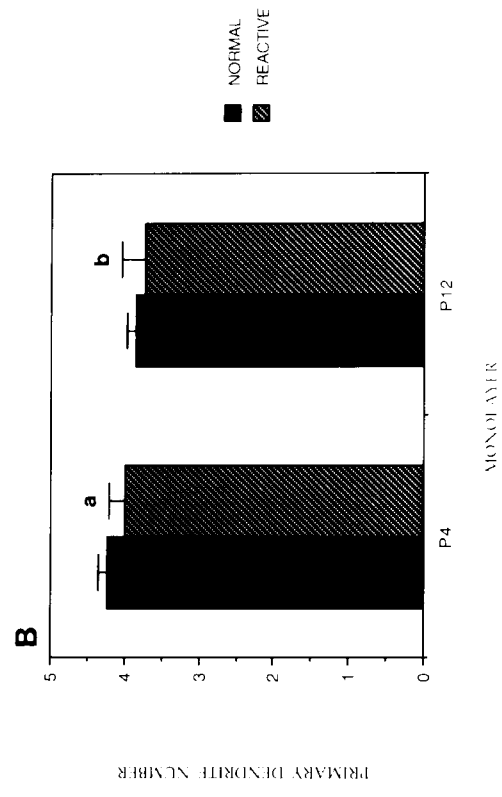
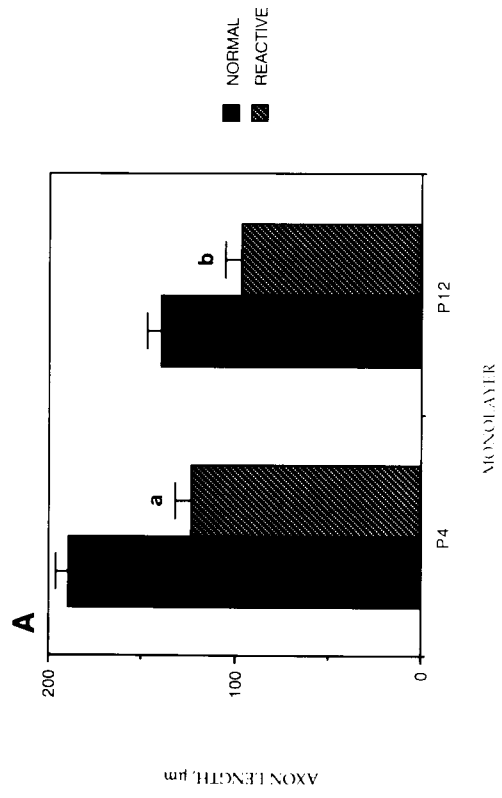


FIGURE 17: Reactive astroglia derived from lesioned cortex support dendrite growth, but demonstrate a reduced ability to promote axon growth from mouse cortical neurons *in vitro*. Histograms illustrating axon length (a), primary dendrite number (b), primary dendrite length (c) and total dendrite output (d) from mouse cortical neurons cocultured with rat astroglia derived from P4 and P12 non-lesioned and lesioned cortex as described in Materials and Methods. Neurite elaboration was examined in 100 randomly selected neurons growing on each glial monolayer using quantitative analysis and double-labeling immunohistochemical techniques. Values represent the mean \pm SEM. Axon length was decreased on both P4 and P12 reactive cortical glial monolayers (a = $p < 0.001$ vs P4 normal; b = $p < 0.01$ vs P12 normal and $p < 0.05$ vs P4 reactive). Total output of primary dendrites was similar on astroglia derived from lesioned or nonlesioned cortex (a = $p > 0.1$ vs P4 normal; b = $p > 0.1$ vs P4 reactive and P12 normal). The number of primary dendrites (a = $p > 0.5$ vs P4 normal and P12 reactive; b = $p > 0.5$ vs P12 normal) and to a lesser extent, elongation of individual dendrites (a = $p > 0.1$ vs P4 normal; b = $p < 0.05$ vs P12 reactive and $p > 0.1$ vs P4 reactive) observed from cortical neurons was similar on normal or reactive cortical astroglial cells.



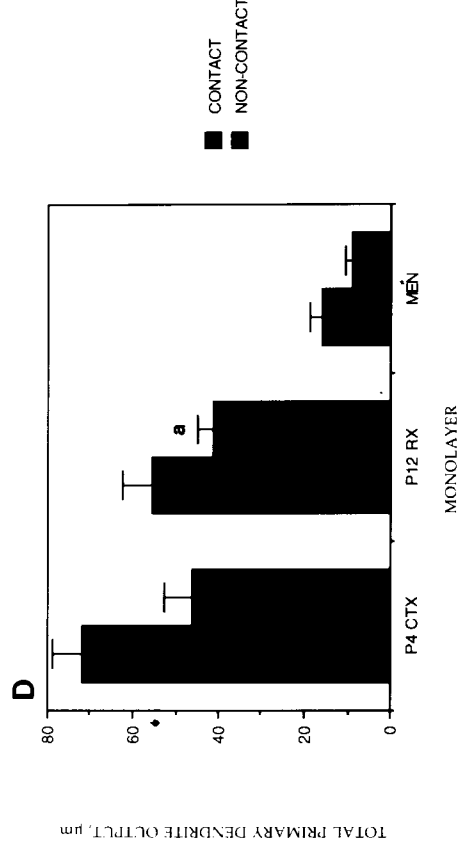
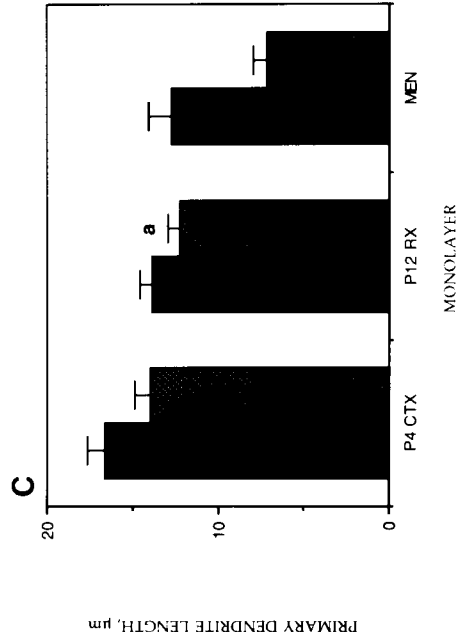
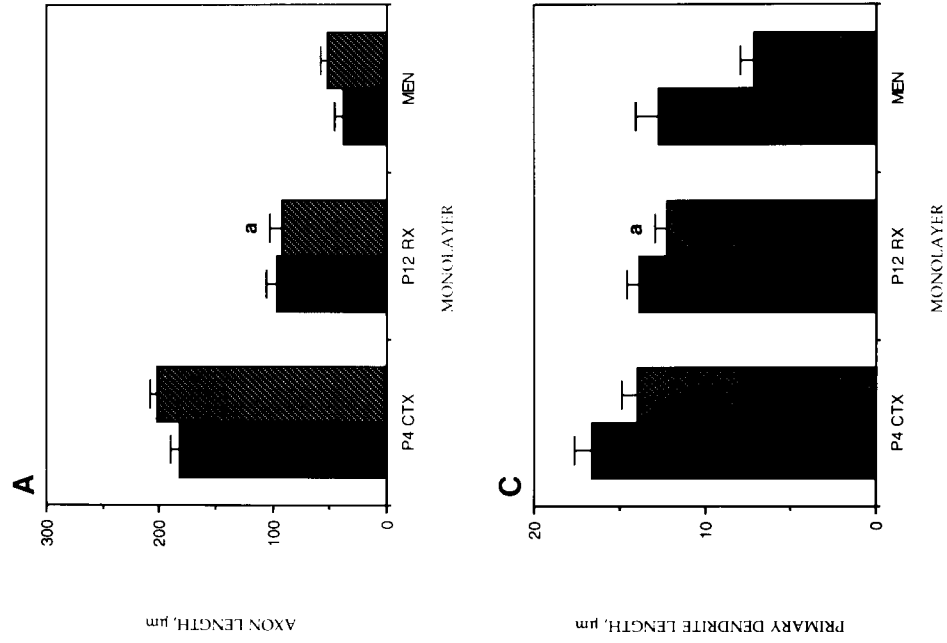
After 5 days *in vitro*, neurons grown directly on P12 reactive astroglia, or in the presence of medium conditioned by P12 reactive astroglia exhibited similar morphological appearances. Primary dendritic outgrowth was slightly less in continuously conditioned medium than direct coculture; however, the small reduction in dendrite growth, between 20 and 30%, was similar to that observed when normal P4 astroglial coculture or noncontact coculture was compared (Figure 18). These observations suggest that reactive astroglial cells may release factors into the culture medium, which support neurite and dendrite growth from cortical neurons.

Fibroblast influence on astroglial supported axon and dendrite growth.

A greater number of fibroblasts, which are known to be poor substrates for neurite growth (Noble et al 1984, Fallon 1985), were observed in monolayers that demonstrated poor support of neurite growth. To determine whether the presence of fibroblasts might explain the observed differences in the ability of mature or reactive astroglia to support process outgrowth, fibroblasts derived from P4 rat meninges, and normal P4 cortical astroglia were mixed in defined ratios in monolayer cultures. Cortical neuron axon and dendrite growth on these monolayers was quantified at 5 days *in vitro*.

Neuron morphology on mixed cultures was similar to the morphology observed on pure P4 astroglial cultures, until fibroblasts constituted greater than 40% of the monolayer. Once this occurred a decrease in total neurite length and primary dendrite number was observed (Figure 19). On P4 astroglial monolayers greater than 90% of neurons elaborated 3 or more dendrites and extended axons greater than 150 μ m in length. By contrast, on monolayers composed of 60% fibroblasts, 28.6% of neurons extended 3 or

FIGURE 18: Cortical neuron dendrite growth is, in part, regulated by a diffusable factor released by astroglia or reactive astroglia. Mouse cortical neurons were grown in a noncontact coculture system in which the medium was continuously conditioned by glial monolayers derived from non-lesioned P4 or lesioned P12 cortex. After 5 DIV axon length (a), primary dendrite number (b), dendrite length (c) and total primary dendrite output (d) was quantified from 100 randomly selected neurons for each astroglial subtype. Results represent the mean \pm SEM. Noncontact and direct coculture of normal P4 astroglia, reactive P12 astroglia and P4 meninges are compared. Axon growth was similar in either contact or noncontact coculture systems of each monolayer tested, but varied depending on the cellular source of the monolayer (a = $p > 0.5$ vs P12 contact; $p < 0.01$ vs men non contact; $p < 0.001$ vs P4 ctx non contact). Primary dendrite elaboration was slightly less prolific in continuously conditioned normal or reactive astroglial medium than direct coculture. However, greater total primary dendrite output (a = $p < 0.001$ vs men non contact; $p > 0.1$ vs P12 RX contact and P4 ctx non contact); an increase in the number of primary dendrites (a = $p < 0.001$ vs men non contact; $p > 0.05$ vs P12 RX contact and P4 ctx non contact) and an increase in the length of primary dendrites (a = $p < 0.01$ vs men non contact; $p > 0.1$ vs P12 RX contact and P4 Ctx non contact) was still observed in the presence of medium continuously conditioned by P12 reactive astroglia when compared with noncontact meningeal coculture (Ctx = cortex, RX = reactive astroglia from injured cortex, MEN = fibroblasts derived from meninges).



more dendrites and 14.7% of axons were greater than 150 μ m in length. Cortical neurons grown on pure meningeal monolayers usually exhibited a unipolar or bipolar morphology. Individual processes were very short and tended to fasciculate; all axons were less than 100 μ m in length and only 24% of primary dendrites were greater than 15 μ m in length.

The relative proportion of fibroblasts to astroglia observed in reactive or mature astroglial monolayers was less the number of fibroblasts observed to decrease neurite growth on P4 astroglia. Therefore, the reduced axon length observed when cortical neurons are grown on P4 and P12 reactive astroglia and the reduction in primary dendrite length observed on P12 reactive astroglia, probably represent an inability of the reactive astroglia to support process growth, rather than attenuation of neurite growth by fibroblasts.

Neuron survival.

A critical point in the interpretation of our results is possible selective survival of neurons, rather than astroglial supported neurite outgrowth; therefore, we determined neuron survival at 5 days *in vitro*. Although neuron survival at 5 days *in vitro* varied among the monolayers prepared from various CNS sources; from 33% on mature mesencephalic astroglia to 52% on mature cortical astroglia ($p < 0.001$; Figure 20); this variation did not clearly correlate with the ability of normal or reactive astroglia to support either axon or dendrite growth.

FIGURE 19: The reduction in neurite growth observed on reactive or mature glial monolayers represents a reduced ability of mature or reactive astroglia to support process growth rather than fibroblast mediated attenuation of neurite growth. Line graphs illustrating axon length (a), primary dendrite number (b), primary dendrite length (c) and total dendrite output (d) from mouse cortical neurons cocultured with P4 rat meningeal fibroblasts and cortical astroglia mixed in defined ratios. Neurite elaboration was examined in 50 randomly selected neurons growing on each monolayer using quantitative analysis and double-labeling immunohistochemical techniques. Values represent the mean \pm SEM. A decrease in axon length, primary dendrite number and total dendrite output was observed when the percentage of fibroblasts in the monolayer exceeded 40% of the cells present. The length of individual primary dendrites decreased when monolayers contained greater than 60% fibroblasts. This proportion of fibroblasts necessary to attenuate neurite growth is greater than the number of fibroblasts observed in purified astroglial cultures.

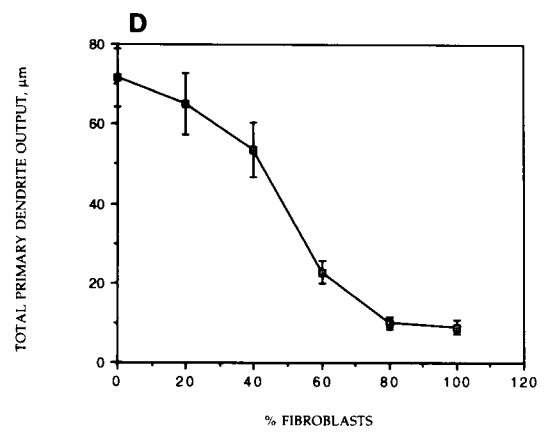
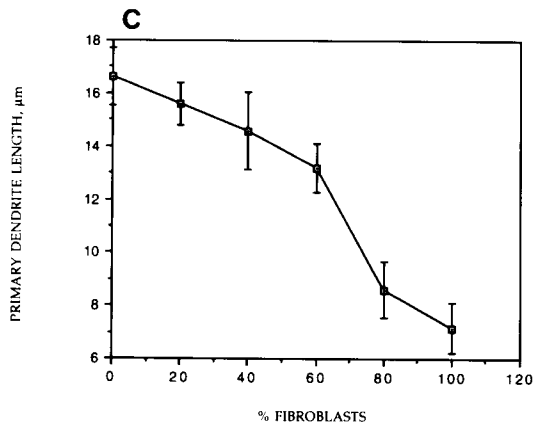
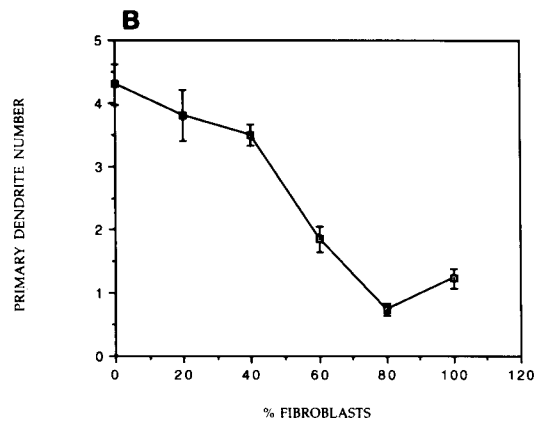
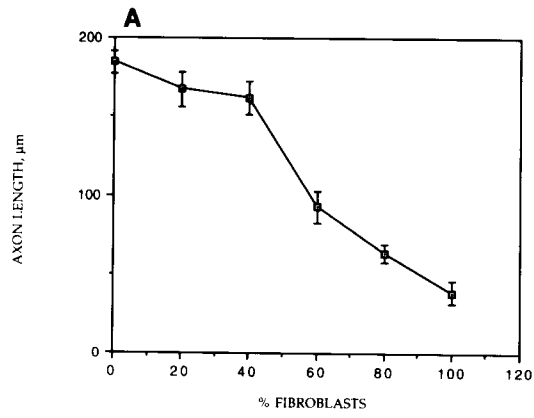


FIGURE 20: Cortical neuron survival varies among different glial monolayers but does not clearly correlate with the ability of the astroglia to support process outgrowth. Neurons were plated at 1×10^4 cells/ml in the presence of various CNS astroglia as described in Materials and Methods and neuron viability determined using morphologic and immunohistochemical techniques at 5 DIV. Values for neuron survival are expressed as the percentage of the original number of viable neurons at 3 hours in culture and represent the mean \pm SEM. Five random microscopic fields on 5 coverslips were examined in three separate cultures of each astroglial subtype. Although variance was observed among the various astroglial subpopulations (ANOVA $p < 0.001$), the extent of dendritic or axon elaboration did not appear to correlate with this effect. (Mes = mesencephalon, Ctx = cortex, RX = reactive glia from injured cortex)

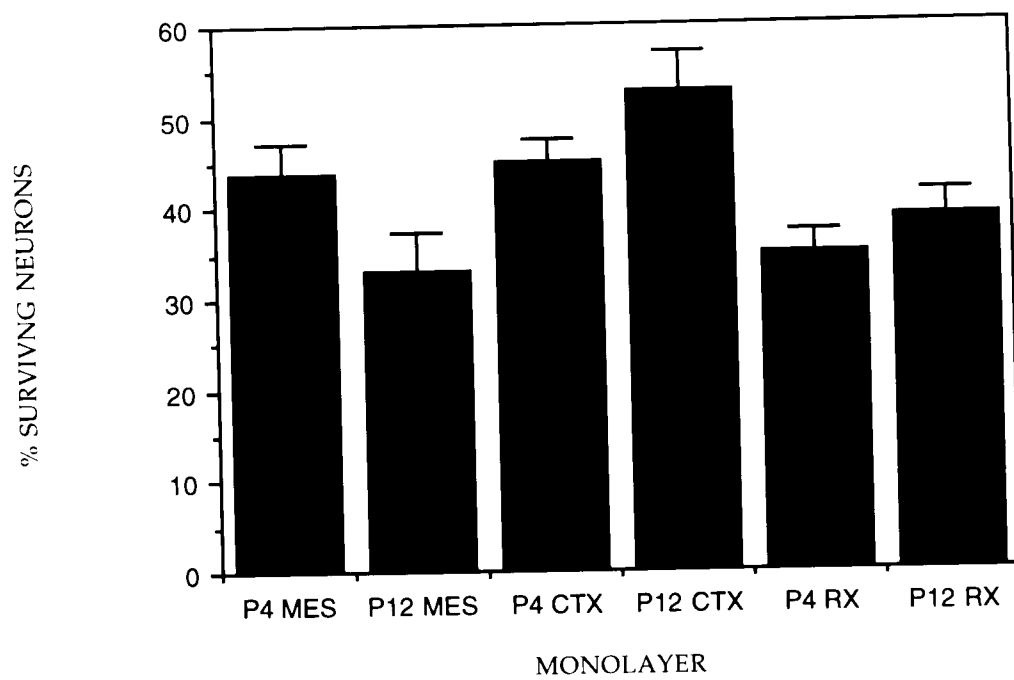
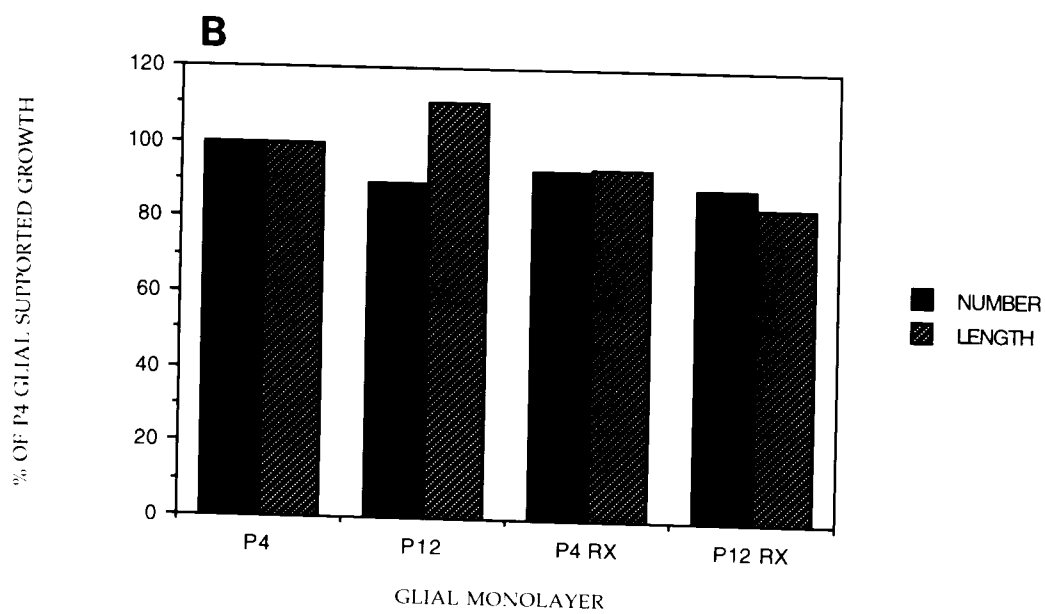
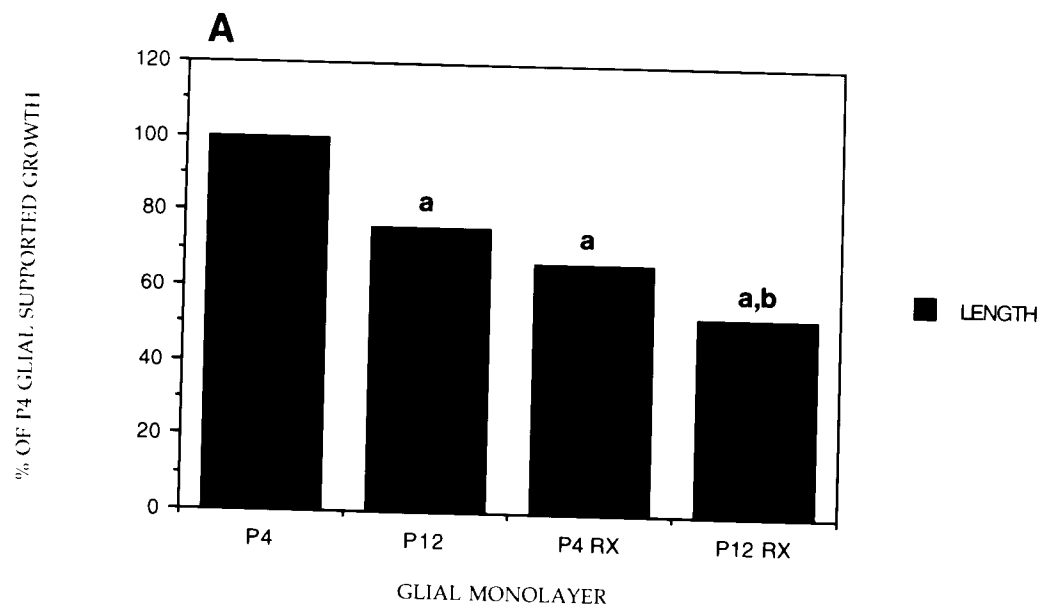


FIGURE 21: Dendrite growth is similar when embryonic cortical neurons are cultured on neonatal, mature or reactive cortical astroglia. By contrast, axon growth is significantly reduced when cortical neurons are cultured on mature or reactive astroglia. Histograms illustrating axon length (a), primary dendrite number and primary dendrite length (b) from mouse cortical neurons cocultured with rat astroglia derived from P4 and P12 non-lesioned or lesioned cortex. Values represent the percentage of mean growth observed on normal P4 cortical astroglia. Neurite elaboration was examined in 100 randomly selected neurons growing on each glial monolayer after 5 DIV using quantitative analysis and double-labeling immunohistochemical techniques. (RX = reactive astroglia obtained from lesioned cortex; a = $p < 0.001$ vs P4 cortex and b = $p < 0.01$ vs P12 cortex)



DISCUSSION

The data reported here (summarized in figure 21) are consistent with, and extend previous observations that mature and reactive astroglia have a reduced ability to support neurite growth *in vitro*. Our results suggest that mature and reactive astroglia are able to support dendrite growth, whereas their ability to support axon growth is reduced. A mechanism that allows separate regulation of axon and dendrite growth may have important implications in CNS regeneration, re-establishment of synaptic contacts and functional recovery after injury to the mature mammalian brain.

Mature astroglia support dendrite growth but demonstrate a reduced ability to support axon growth.

The results in this study suggest that primary dendrite, but not axon growth is supported by mature astroglia. This observation is consistent with previous investigations, both *in vitro* and *in vivo*, indicating that mature astrocytes, derived from rats older than postnatal day 8, have a reduced capacity to support neurite growth when compared with immature astrocytes (Smith et al 1986 and 1990). The difference, however, is almost entirely explained by the reduction in length of the longest process, i.e. the axon, on mature astroglia. It is possible that astrocyte differentiation, but not necessarily chronological age may determine its relative ability to support axon or dendrite growth (Hatten et al 1991). For example, in the developing mammalian brain, radial glia, rather than differentiated astrocytes, act as guide posts for axon growth (Rakic 1988, Norris and Kalil 1991). Expression of MAP2, a dendrite specific marker, is not found during the time radial glia are observed in the brain (Crandall et al 1986); suggesting perhaps that radial glia do not support dendrite growth.

Dendritic architecture may never reach a mature or stable state in the adult mammalian nervous system (Purves et al 1986, Coleman and Flood 1986, Petit et al 1988). Furthermore, there is evidence that dendritic morphology can remodel and grow in response to the environment and release of neurotransmitters (Coleman and Flood 1986, Mattson et al 1988 and 1989, Popov et al 1992). For example, dendritic proliferation is observed in regions of the brain that demonstrate age related neuronal loss both in animal models of aging and the human brain (Coleman and Flood 1986). Previous observations *in vitro* have demonstrated that astroglia can selectively modulate the development of primary dendrites (Denis-Donini et al 1984, Chamak et al 1987, Rousset et al 1988 and 1990, chapter 9); therefore it is perhaps not surprising that mature astrocytes may also provide support of dendrite elaboration. Subsequent refinement of the dendritic tree, however, may depend on neuronal activity or other factors in the environment.

Reactive astroglia in the immature and mature mammalian brain.

Astrocytes respond to a variety of insults to the CNS by rapid and vigorous reactive astrogliosis. Reactive astrogliosis is observed in acute conditions such as trauma or stroke, around neoplasms and also in neurodegenerative conditions such as Alzheimer's disease (Hatten et al 1991, Eddlestone and Mucke 1993). Studies of injury in the young mammalian brain demonstrate that reactive astrogliosis can occur and is present within 2-4 days of injury (Berry et al 1983, Barrett et al 1984, Moore et al 1987, Trimmer and Wunderlich 1990, Balasingam et al 1994). Injuries sustained during the embryonic or neonatal period, however, produce less astrogliosis relative to similar injuries in the adult (Dahl and Bignami 1976, Berry et al 1983, Barrett

et al 1984, Smith et al 1986, Rudge et al 1989, Rudge and Silver 1990, Balasingam et al 1994). Our results suggest that one of the functional consequences of reactive astroglia, reduced support of axon growth, is a feature of reactive astroglia in general and not necessarily the severity of the reaction.

Reactive astroglia support primary dendrite number but not axon growth.

The relationship of the neuroglial scar, in particular the reactive astrocyte and the failure of CNS regeneration is often linked (Berry et al 1983, Fishman et al 1983, Reier et al 1983, Liuzzi and Lasek 1987); however, several recent experimental observations indicate that reactive astrocytes may, in some circumstances, support process outgrowth (Gage et al 1988, Anders and Johnson 1990, David et al 1990 Hatten et al 1991, Kawaja and Gage 1991). Our results suggest the interesting possibility that reactive astroglia are able to maintain dendrite growth, principally primary dendrite number. By contrast, axon elongation is reduced on both neonatal and mature reactive astroglia. These observations are consistent with previous *in vitro* investigations indicating that total neurite growth is reduced on reactive astroglia, particularly if the astroglia are matured in culture (Rudge and Silver 1990, McKeon et al 1991). The difference in growth, however, is almost entirely accounted for by the reduction in support for the longest neurite, suggesting that dendrite growth appears to be equally supported on reactive astroglia of different ages. A role for reactive astroglia in regeneration is consistent with tissue culture studies that consistently demonstrate that astroglia are an excellent substrate for process outgrowth and appear to regulate axon and dendrite growth separately (Denis-Donini et al 1984, Chamak et al 1987, Rousset et al 1988 and 1990, chapter 9).

How do reactive astrocytes support dendrite growth? The production of astrocyte soluble peptide growth factors such as insulin like growth factor 1 (Garcia Estrada et al 1992), nerve growth factor (Bakhit et al 1991), epidermal growth factor (Nieto-Sampedro et al 1988) and fibroblast growth factor (Finklestein et al 1988, Gomez-Pinilla et al 1992, Logan et al 1992) is increased after cortical lesions (Nieto-Sampedro et al 1982, Manthorpe et al 1983). The biologic significance of astrocyte released growth factors in the cascade of cellular events that occur after injury is uncertain; however, soluble peptide growth factors may mediate neuron protection (Vilbulsreth et al 1987, Mattson and Rychlik 1990, Cheng and Mattson 1991, Yamada et al 1991, Frim et al 1993, Maeise et al 1993), neurite outgrowth (Hatten et al 1988, Walicke et al 1988, De Konnick et al 1993) or attenuate the deleterious effects of excitatory amino acids on dendrite growth (Mattson et al 1989). This latter action may be particularly significant since excitatory amino acids are considered integral to the pathophysiology of neuronal injury and death in a variety of neurologic disorders (Lipton and Rosenberg 1994). There is considerable evidence that normal astroglial cells secrete diffusible neurite promoting factors, (Hatten et al 1988, Rousset et al 1988 and 1990, chapter 9) which, in part, mediate dendrite growth independently from axon growth. Locally produced astroglial growth factors may be better able to support dendrite growth, whereas poor axonal growth may result from a lack of target derived neurotrophic factors from distant neuronal cell bodies.

What accounts for limited growth of axons observed on reactive astroglia?

Reactive astroglia have been postulated to create a mechanical barrier to process growth (Reier et al 1983). Analysis of the glial scar cellular

environment *in vivo*, however, demonstrates many additional factors that may contribute to decreased axon growth. For example, the glial scar, in addition to astrocytes is composed of a complex mixture of microglia, oligodendrocytes, neutrophils, macrophages and fibroblasts (Berry et al 1983, Matthewson and Berry 1985, Giulian et al 1989, Moreno-Flores et al 1993); each or these cell types may contribute to reduced axon growth (Fallon 1985, Schwab and Caroni 1988, Rudge and Silver 1990, Savio and Schwab 1990, Schnell and Schwab 1990, McKeon et al 1991, Johnson-Green et al 1992, Giulian et al 1993, Thanos et al 1993). Too few of these cell types were present in our culture system for this to be a significant factor. We cannot, however, exclude that relatively small numbers of cells, such as microglia or oligodendrocytes, were able to release sufficient quantities of inhibitory substances to attenuate reactive astroglial support of process growth.

Oligodendrocytes and myelin products can attenuate axon growth. For example, when sections of rat CNS are used as substrata for peripheral neuron growth, neurite elongation is possible on gray but not white matter (Savio and Schwab 1989, Crutcher 1989). In addition, when antibodies raised to proteins eluted from white matter (Caroni and Schwab 1988) are applied to transected nerves *in vivo* spinal cord regeneration can occur (Schnell and Schwab 1990). Similarly if oligodendrocytes are first eliminated by radiation, corticospinal axon growth is observed in the transected spinal cord (Savio and Schwab 1990).

It is unlikely, however, that the few oligodendrocytes identified in the various glial monolayers contributed to the experimental observations. First, the reactive astroglia were obtained from gray matter which does not contain

the inhibitory molecules associated with oligodendrocytes (Caroni and Schwab 1988). Second, oligodendrocytes constituted less than 2.5% of the cells identified in the monolayers. In mixed cultures an oligodendrocyte to astrocyte ratio of least 6:1 does not modify the pattern of neurite growth supported by astroglia (Ard et al 1991). Purified cortical astroglia, or their conditioned medium, have also been observed to inhibit myelination and decrease oligodendrocyte proliferation and survival *in vitro* (Rosen et al 1989). Finally, several experiments demonstrate that axonal regeneration is limited even in wounds where myelin or oligodendrocytes are not a major component (Carlstedt et al 1985), such as the nonmyelinated retina (McConnell and Berry 1982). In addition, Schwab and colleagues have acknowledged that even in the presence of myelin protein antibodies, the majority of axons fail to regenerate in transected nerves (Schnell et al 1993). Oligodendrocytes and myelin products may therefore only be relevant in injuries where they are present in large quantities such as when white matter is involved.

Microglia are among the first cells to respond to CNS injury (Kitamura et al 1978) and are postulated to stimulate astrogliosis (Giulian and Baker 1985, Giulian and Lachman 1985, Giulian et al 1988) or the release of other growth factors by astrocytes (Vige et al 1991). Reactive astrocytes, however, can also be observed in the absence of microglia or before microglial accumulation (Steward et al 1990, Mucke et al 1991). For example, cessation of neuronal activity (Canady and Rubel 1992) or alterations in the extracellular milieu, including the accumulation of glutamate (Petito et al 1991), can initiate reactive astrogliosis.

Can microglia contribute to the limited axonal growth observed on reactive astroglia? Several lines of evidence suggest it is unlikely that the microglia observed in our experiments had either a direct or indirect influence on axon growth. First, for microglia to modify astrocyte function, a greater density of microglia than were found in our experiments is required (Johnson-Green et al 1992). Few microglia were observed because: 1) simple penetrating injuries to the CNS appear to be accompanied by a limited microglial response (Rio-Hortega 1932, Berry et al 1983, Balasingam et al 1994), and 2) the culture techniques used to produce the reactive glial monolayers are similar to those used by other investigators that reliably yield cultures from normal or injured brain that are up to 99% pure for astrocytes with few if any microglia detectable by techniques such as morphology, ultrastructural analysis and immunohistochemistry (Morrisson and DeVellis 1981, Kimelberg 1983, Raff et al 1988, Hansson 1988, Ingraham and McCarthy 1989, Rudge et al 1989, Kliot et al 1990, Wesselingh et al 1990, O'Malley et al 1992, Schwartz and Marini 1992, Schwartz and Wilson 1992, Wang et al 1994). Second, microglia may not be active unless activated by mediators of inflammatory reactions (Giulian and Baker 1985, Heiter et al 1988 and 1990, Johnson-Green et al 1992, Giulian et al 1993). In addition, the release of microglial cytokines *in vitro*, which can affect the astrocytic reaction, is also only observed after stimulation by mediators of inflammatory reactions (Johnson-Green et al 1992, Giulian et al 1993). Since these substances were not present in our culture system, it is unlikely that the few microglia identified were active or released cytokines. Finally, microglia appear to contribute to neurotoxicity both *in vitro* and *in vivo* (Thanos 1991, Giulian and Robertson 1990, Giulian 1993, Thanos et al 1993). For example, cell culture studies indicate that microglia release a variety of cytotoxic agents, including free radicals that contribute to neuronal

death (Giulian and Baker 1986, Giulian et al 1993). Neuronal death often stimulates axonal sprouting (Steward 1989). In addition, neuron survival on monolayers derived from injured brain was not adversely affected suggesting that reactive astroglia rather than microglia were the dominant cell type in the glial monolayers.

The most likely explanation for reduced axon growth observed on reactive astroglia is secondary to intrinsic cellular changes such as the expression of nonpermissive isoforms of growth promoting molecules and the expression of growth inhibiting molecules by the reactive astroglia. Increased expression of putative inhibitory molecules, which alone or in combination may inhibit elongation of neurites, have been observed in the injured brain. For example, increased immunoreactivity for tenascin and chondroitin sulfate proteoglycan (CS-PG) have been observed at sites of glial injury or in explants of cortical wounds derived from older animals (McKeon et al 1991, Laywell et al 1992). In the normal developing CNS both tenascin and CS-PG play an important role in restricting processes to the appropriate position (Snow et al 1990a and 1990b, Steindler 1993). In the setting of injury, however, overexpression of inhibitory molecules may prevent regrowth of axons (McKeon et al 1991). This may have important functional consequences since inappropriate axonal connections may often be deleterious to CNS function. Continued astroglial support of primary dendrite growth, however, may enhance synaptic connections and provide a framework on which neuronal activity can refine neural structure. Astroglial mediated coordination of axon and dendrite growth in the mature or injured CNS may therefore represent a critical factor in the establishment of appropriate neuronal connections, adaptation to the environment and restoration of function.

**THE MOLECULAR BASIS OF DENDRITE GROWTH: A PRELIMINARY
ANALYSIS OF ASTROGLIAL CONDITIONED MEDIA AND REVIEW OF
POTENTIAL MECHANISMS OF ASTROGLIAL MEDIATED DENDRITE
GROWTH**

INTRODUCTION

The establishment, development and maintenance of CNS neuron morphology and function, including the elaboration of axons and dendrites, depends, in part, on neuron-glial interactions. Neuron-glial interactions are mediated by a tremendous diversity of factors including soluble trophic molecules, molecules linked to cell membranes such as cell adhesion molecules, components of the extracellular matrix, physical parameters such as adhesion, and second messenger systems (Dodd and Jessel 1988, Barde et al 1989, Lipton 1989, Sanes 1989, Crossin et al 1990, Bixby and Harris 1991, Reichardt and Tomaselli 1991, Thiery and Boyer 1992, Letourneau et al 1994). Each class of molecules or factors has been observed to influence major steps in neuron development including survival, migration, differentiation, axon growth and guidance, and synapse formation.

Astroglia can modulate dendrite elaboration; the precise molecular nature of the factor or factors responsible for astroglial mediated dendrite growth, however, is not known. Diffusible protein factors released by astroglia (Rousselet et al 1988 and 1990, Seil et al 1992), and soluble components of the extracellular matrix associated with the surface of astrocytes (Rousselet et al 1988 and 1990, LaFont et al 1992) have both been implicated in the morphogenetic process of cerebellar or mesencephalic neurons in culture. In

other tissue culture studies, previously characterized peptide growth factors or purified extracellular matrix molecules that are known to be expressed by astrocytes have also been observed to support dendrite growth from sympathetic, sensory or mesencephalic neurons (Chamak and Prochiantz 1989, Lein and Higgins 1989, LaFont et al 1992, De Koninck et al 1993), or attenuate glutamate mediated dendrite regression in hippocampal neurons (Mattson et al 1989).

Our earlier experimental observations demonstrated that cortical neuron dendrite growth is supported by astroglia that continuously condition the culture medium (chapter 9). In this preliminary study we asked what diffusible factor or factors released by astroglia may be responsible for mediating dendrite growth. To address this question we used protein biochemical techniques and a tissue culture bioassay to determine the following: 1) does astroglial conditioned medium demonstrate regional heterogeneity supporting dendrite growth and release of corresponding diffusible factors, 2) what are the biochemical characteristics of dendrite supporting medium, and 3) which previously characterized soluble peptide growth factors can support dendrite growth? The preliminary biochemical findings provide necessary background for the identification and purification of molecules that can promote dendrite growth in the CNS.

MATERIALS AND METHODS

The experimental methods are described in detail in chapter 7. Briefly, cortical neurons prepared from E18 mouse embryos were plated at low density on poly-D-lysine coated coverslips in: 1) astroglial conditioned medium obtained

from purified confluent mesencephalic or cortical glial monolayers, 2) conditioned medium that had been subjected to protein denaturation, molecular weight fractionation or heparin affinity chromatography, and 3) chemically defined medium to which soluble peptide growth factors were added. The growth of neurites from cortical neurons, identified by M6, NF-H or MAP2 immunoreactivity, was quantified after 5 days *in vitro*. Three experiments for each condition were carried out. A total of 30 double-labeled immunoreactive neurons were examined in each experimental condition. In addition, ^{35}S methionine radiolabeled protein samples were extracted from astroglial monolayers derived from various CNS regions and astroglial conditioned medium and subjected to SDS-PAGE electrophoresis.

RESULTS

Neurite outgrowth in astroglial conditioned medium.

We have previously observed that neurons grown in an astroglial non-contact coculture system elaborate dendrites depending on the CNS region from which the astroglia are derived (chapter 9). To confirm that astroglia release diffusible factors that regulate dendrite growth, we plated E18 cortical neurons at low density on polylysine coated coverslips in the presence of medium conditioned by cortical or mesencephalic astroglial monolayers. After 5 DIV axon and dendrite growth was quantified using morphologic and double-labeling immunohistochemical techniques. Table 9 summarizes the extent of axon and dendrite elaboration observed in coculture, non-contact coculture, or conditioned medium (CM). Figure 22 illustrates the main morphological differences observed between cortical neurons cultured in medium conditioned by astroglia derived from the cortex or mesencephalon.

TABLE 9: CORTICAL NEURON DENDRITE GROWTH IN ASTROGLIAL CONDITIONED MEDIUM

| Astroglial condition | Axon length, μm | Primary dendrite length, μm | Primary dendrite number | Total primary dendrite output, μm |
|--------------------------------------|----------------------------|--|-------------------------|--|
| Cortex, coculture | 184.7 \pm 7.5 | 16.6 \pm 1.1 | 4.3 \pm 0.3 | 71.7 \pm 7.2 |
| Cortex non-contact coculture | 201.5 \pm 7.2 | 14.0 \pm 1.0 | 3.3 \pm 0.3 | 46.2 \pm 6.6 |
| Cortex, conditioned medium | 172.5 \pm 6.4 a | 18.9 \pm 0.7b | 3.3 \pm 0.3c | 61.7 \pm 5.9c |
| Mesencephalon, coculture | 181.4 \pm 6.5 | 16.1 \pm 1.8 | 1.4 \pm 0.1 | 22.6 \pm 3.3 |
| Mesencephalon, non-contact coculture | 187.1 \pm 7.7 | 13.3 \pm 2.0 | 1.3 \pm 0.2 | 18.6 \pm 4.5 |
| Mesencephalon, conditioned medium | 170.9 \pm 8.5 | 13.6 \pm 1.4 | 1.3 \pm 0.3 | 17.2 \pm 5.5 |

Table 9: Cortical neuron dendrite growth in astroglial conditioned medium depends on the CNS source of the astroglia and is comparable to dendritic growth observed in direct coculture with the glial monolayer. Embryonic mouse cortical neurons were grown in direct coculture, a noncontact coculture system or in medium conditioned (CM) by astroglia derived from the mesencephalon or cortex for 5 days. Axon length, primary dendrite number, primary dendrite length and total primary dendrite output was quantified from 100 randomly selected neurons from three experiments for each astroglial subtype. Results represent the mean \pm SEM. (a) $p>0.05$ compared with mesencephalon CM ; b) $p=0.001$ compared with mesencephalon CM; c) $p<0.001$ compared with mesencephalon CM).

Dendrite growth was influenced by the CNS region from which the astroglial CM was derived (Table 9). In medium conditioned by cortical astroglia, the majority of cells, 72.9%, elaborated 3 or more primary dendrites. By contrast, the majority of neurons grown in medium conditioned by mesencephalic astroglia were unipolar or bipolar; only 21% of neurons elaborated 3 or more primary dendrites ($p < 0.001$). Neurons grown in medium conditioned by cortical astroglia extended longer primary dendrites ($p < 0.01$) and elaborated a more extensive total primary dendrite arbor ($p < 0.001$) than neurons grown in mesencephalic astroglial CM.

Axon length did not differ significantly in medium conditioned from various CNS regions; 69.3% of axons examined in cortical astroglial CM were greater than 150 μ m in length, whereas 63% of neurons extended axons greater than 150 μ m in mesencephalic astroglial CM (Table 9).

Protein factors mediate neurite growth.

To determine the molecular nature of the factors present in cortical astroglial CM that support dendrite growth, protein in the medium was denatured by boiling or trypsin treatment. To ensure optimal neuron survival, treated medium was supplemented with 20% of chemically defined medium. Embryonic (E18) cortical neurons were plated at low density on polylysine coated coverslips. Both heat inactivation and trypsin treatment reduced the cortical astroglial CM induced neurite elaboration and decreased the number of primary dendrites elaborated by neurons (Figure 22 and 23). In addition, neurons grown in treated medium elaborated shorter axons than neurons grown in CM. These results suggest that the factors present in the cortical

FIGURE 22: Cortical neurons growing in astroglial conditioned medium (CM) *in vitro* exhibit dendritic differences depending on the treatment of the medium. Representative fluorescent videophotomicrographs of E18 mouse cortical neurons grown in mesencephalic CM (a) cortical CM (b), trypsin treated cortical astroglia CM (c), heparin bound cortical CM fraction (d), cortical CM fraction containing molecules greater than 100kDa (e) and cortical CM fraction containing molecules between 10 and 100kDa (f), and labeled with anti-M6 after 5DIV. The majority of neurons extended multiple neurites in cortical CM, its heparin bound fraction and the fraction containing molecules between 10 and 100kDa. By contrast, in medium conditioned by mesencephalic astroglia, cortical CM denatured by trypsin or greater than 100kDa, the majority of neurons were uni or bipolar. (Scale bar = 10 μ m)

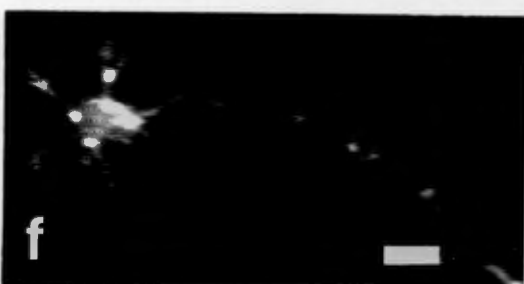
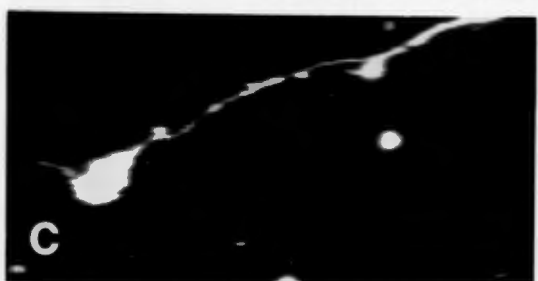
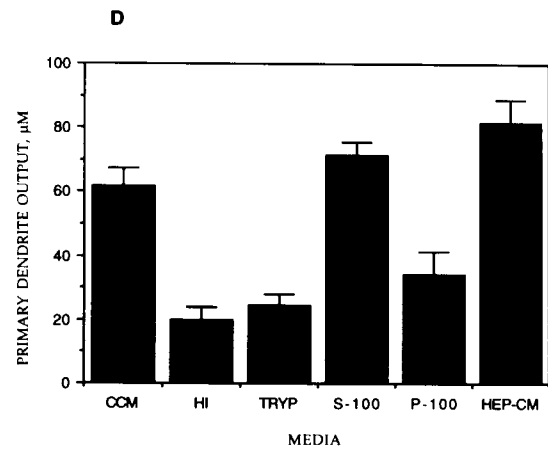
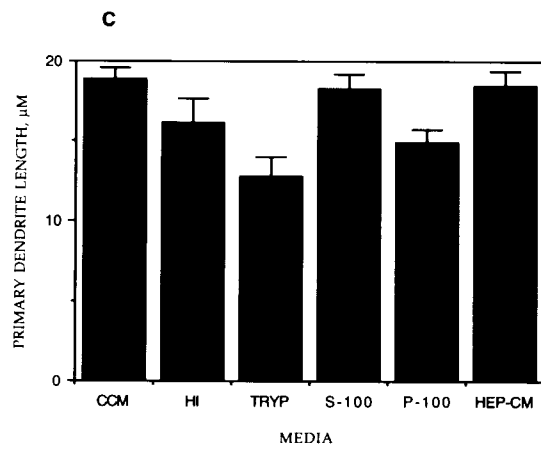
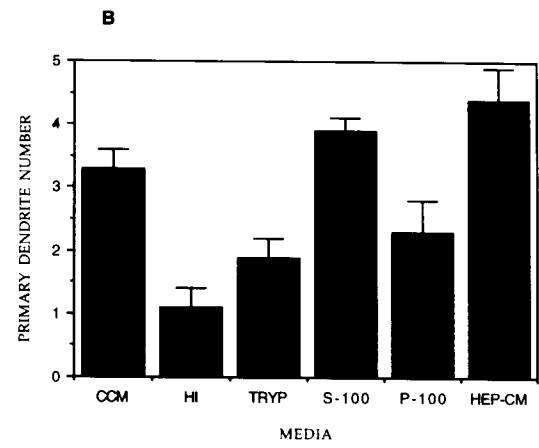
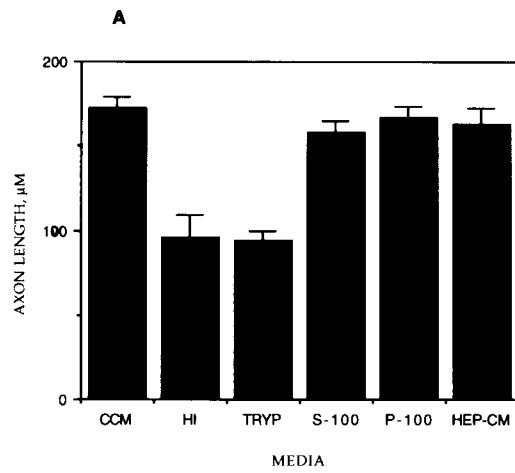


FIGURE 23: The treatment of cortical astroglial conditioned medium (CM) influences the extent of the primary dendritic arbor elaborated by cortical neurons in culture. Histograms illustrating axon length (a), primary dendrite number (b), primary dendrite length (c), and total dendrite output (d) from mouse cortical neurons cocultured in cortical astroglial CM treated as described in Materials and Methods. Neurite elaboration was quantitatively examined in 30 randomly selected neurons growing in each conditioned medium using morphologic and double-labeling immunohistochemical techniques. Values represent the mean \pm SEM. (CCM = cortical astroglial conditioned media, HI = heat inactivated CCM, TRYP = trypsin treated CCM, S-100 = 10 to 100kDa CCM fraction, P-100 = CCM fraction greater than 100kDa, HEP-CM = heparin bound fraction of cortical astroglial CM)



medium that mediate both axon and dendrite growth are proteins, or require protein cofactors.

Astroglial conditioned medium derived from various CNS sources contain different proteins.

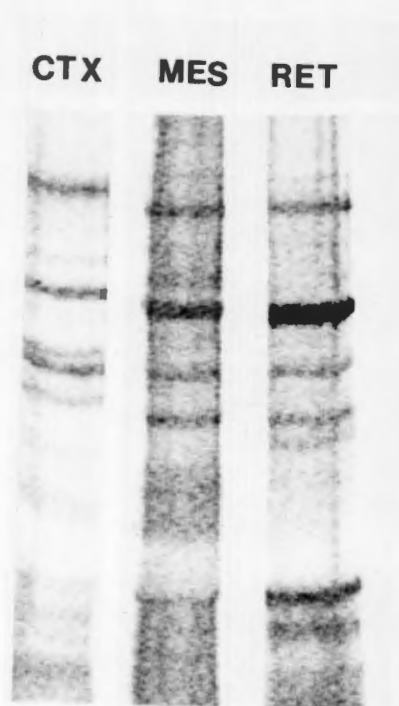
Protein gel electrophoresis was performed to determine whether astroglia derived from different CNS regions released different proteins into the culture medium. Glial monolayers and proteins released into the medium were labeled with ^{35}S methionine. The electrophoretic profile of ^{35}S methionine labeled proteins extracted from astroglia of various CNS sources is illustrated in figure 24. Conditioned medium demonstrated different protein bands depending on the CNS region from which the astroglia were derived (Figure 24). By contrast, radiolabeled protein extracted from the various glial monolayers demonstrated markedly similar protein profiles. A single protein that was common to dendrite supporting medium, such as cortical or retinal astroglial CM, but was not visible in non-supporting dendrite medium, could not be identified (Figure 24). These results suggest that more than one protein factor present in astroglial CM may be capable of supporting dendrite growth.

Factors between 10kDa and 100kDa support dendrite growth.

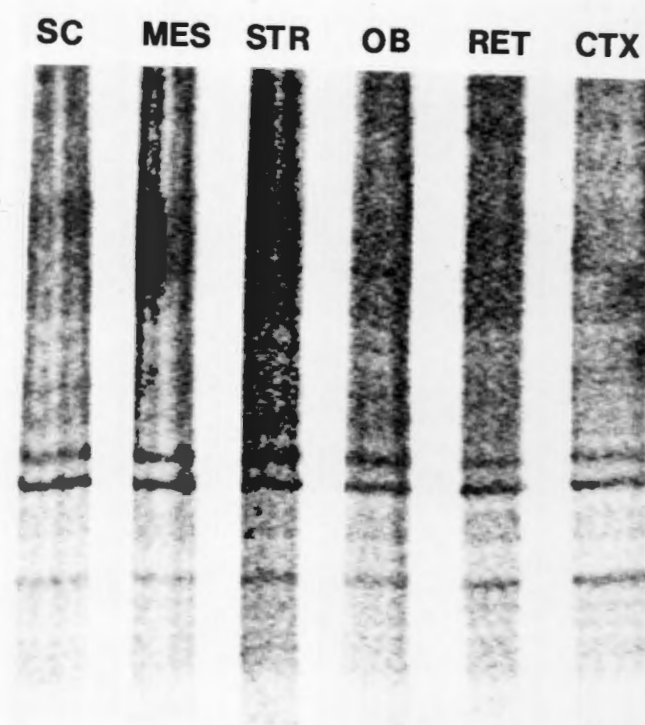
To determine the molecular weight of the factor present in medium conditioned by cortical astroglia that promotes process growth, CM was separated by ultrafiltration and dialysis into three molecular weight fractions: 1) less than 10kDa, 2) between 10 and 100kDa, and 3) greater than 100kDa. Embryonic cortical neurons were plated at low density on polylysine and grown in each media fraction.

FIGURE 24: Conditioned medium (CM, a), but not protein extracted from the various glial monolayers (b), demonstrated different protein bands depending on the CNS region from which the astroglia were derived. Proteins were labeled with ^{35}S Methionine and samples subject to SDS-PAGE electrophoresis as described in Materials and Methods. A single protein that was common to dendrite supporting CM, such as cortex or retina, but not observed in dendrite non-supporting CM, such as mesencephalic CM, however, was not observed. (Fib = fibroblasts from meninges, SC = spinal cord, Mes = mesencephalon, Str = striatum, OB = olfactory bulb, Ret = retina, Ctx = cortex)

A



B



The extent of process outgrowth, after 5 DIV, depended on the molecular weight fraction of the medium (Figure 23). The majority of neurons (80%) grown in the cortical astroglial CM fraction between 10 and 100kDa extended more than three primary dendrites. By contrast, few neurons (28.5%) grown in the CM fraction greater than 100kDa extended more than three primary dendrites. Neuron survival was poor in the CM fraction containing molecules less than 10kDa; the majority of surviving neurons, however, were unipolar and elaborated only a short axon. Mean total primary dendritic arbor was greatest in the CM fraction containing molecules between 10 and 100kDa and was nearly threefold greater than that observed from neurons cultured in CM containing molecules greater than 100kDa. This effect was on both the number and length of individual primary dendrites (Figure 23). In contrast to dendrite growth, axon length was similar in the CM fractions between 10 and 100kDa and the fraction greater than 100kDa; axons in both these fractions, however, were nearly threefold longer than the axons elaborated by neurons grown in the fraction less than 10kDa.

Fibroblast growth factor supports dendrite and axon growth.

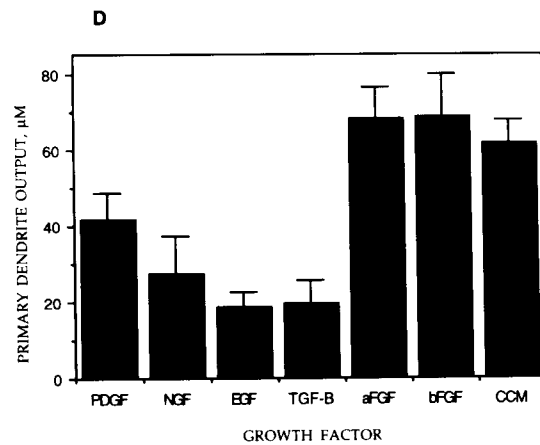
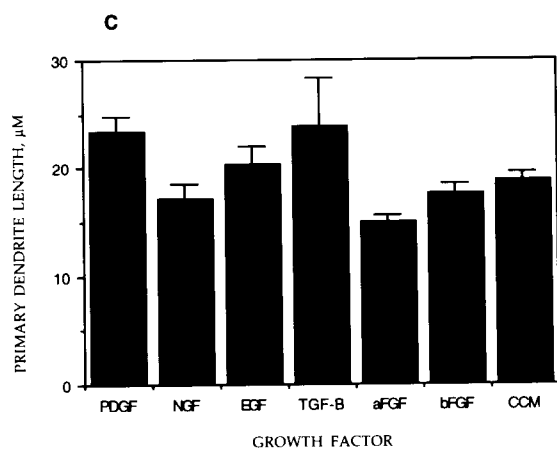
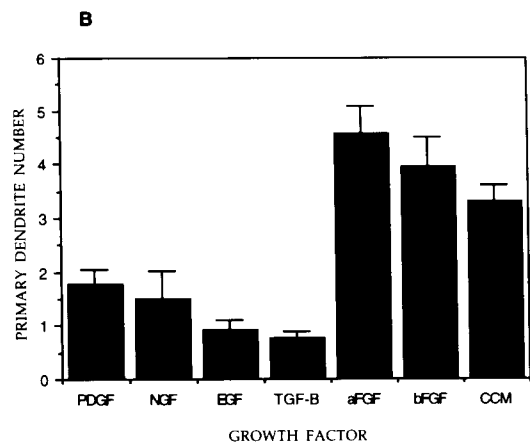
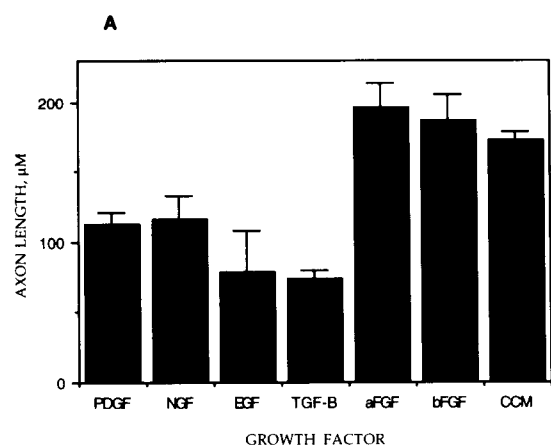
Several previously identified polypeptide growth factors, many of which are between 10 and 100kDa in weight, have been observed to promote neurite outgrowth from CNS neurons (Morrison et al 1986, Walicke et al 1986, Hatten et al 1988). None of these investigations, however, have reported separate quantification of axon or dendrite growth. Are these growth factors able to support dendrite growth? In the next series of experiments we compared the number and length of neurites elaborated by E18 cortical neurons after 5 days in culture in the presence of various growth factors. Neurons were plated at low density (1×10^4 cells/ml) on polylysine coverslips and maintained in

chemically defined medium. Growth factors were added one hour after plating the neurons, the culture medium was changed every two days and fresh growth factors were added at each feeding. Cultures were treated with cytosine arabinoside (AraC; 10 μ M) to eliminate the growth of non-neuronal cells. Heparin (1 μ g/ml) was added to FGF fed cultures to maximize the effect of the growth factor.

Four groups of growth factors were tested: 1) astrocyte expressed growth factors that promote CNS neurite, but not specifically axon or dendrite, growth (aFGF and bFGF; Morrison et al 1986, Ferrara et al 1988, Hatten et al 1988, Woodward et al 1992), 2) astrocyte expressed growth factors that promote neurite growth in the peripheral and sympathetic nervous system (NGF, TGF; Saad et al 1991, Chalazonitis et al 1992, Lazar and Blum 1992, de Konnick et al 1993), 3) growth factors of possible astrocyte origin that promote CNS neurite, but not specifically axon or dendrite, growth (EGF; Fallon et al 1984, Morrison 1987), and 4) growth factors of possible astrocyte origin that do not promote neurite growth (PDGF; Morrison et al 1987, Richardson et al 1988, Yeh et al 1991, Pringle et al 1992). The results of this analysis are illustrated in Figure 25.

After 5DIV embryonic cerebral neurons maintained in the presence of aFGF and bFGF exhibited similar morphologies to neurons grown on cortical astroglial monolayers or in cortical astroglial CM. The majority of neurons grown in the presence of aFGF and bFGF were multipolar and typically extended a long axon and three or more primary dendrites. By contrast, neurons grown in the presence of TGF, EGF, NGF or PDGF were usually uni- or bipolar and extended a relatively short axon. Interestingly, the length of

FIGURE 25: Fibroblast growth factor supports axon and dendrite growth from cortical neurons in culture. Histograms illustrating axon length (a), primary dendrite number (b), primary dendrite length (c) and total dendrite output (d) from mouse cortical neurons cultured in the presence of: PDGF (10ng/ml), NGF (200ng/ml), EGF (10ng/ml), TGF (10ng/ml), aFGF (1ng/ml) and bFGF (1ng/ml). All cultures were treated with cytosine arabinoside (AraC; 10 μ M) and heparin (1 μ g/ml) was added to FGF fed cultures. After 5DIV neurite elaboration was quantitatively examined in 30 randomly selected neurons growing in the presence of each growth factor using immunohistochemical and morphologic techniques. Values represent the mean \pm SEM. (PDGF = platelet derived growth factor, NGF = nerve growth factor, EGF = epidermal growth factor, TGF = transforming growth factor, aFGF = acidic fibroblast growth factor, bFGF = basic fibroblast growth factor, CCM = cortical astroglial conditioned media).



individual primary dendrites from cortical neurons grown in the presence of TGF, EGF, NGF or PDGF were similar or longer than those elaborated by neurons grown in the presence of aFGF and bFGF.

Fibroblast growth factor is probably present in cortical astroglial conditioned medium.

Expression of FGF protein and mRNA is observed in astrocytes *in vivo* and *in vitro* (Ferrara et al 1988, Emoto et al 1989, Woodward et al 1992). To determine whether biologically active FGF is present in medium conditioned by cortical astroglia, CM was eluted on a heparin binding column. FGF is distinguished by strong binding to heparin (Shing et al 1984). The bound fragment was therefore reconstituted in chemically defined medium and fed to embryonic cortical neurons plated at low density on polylysine coverslips.

Extensive dendrite and axon growth comparable to that observed in cortical astroglial CM, or in the presence of FGF, was observed in the heparin bound fraction of cortical astroglial CM (Figure 22 and 23). The majority of neurons (71.4%) elaborated four or more primary dendrites. Axons greater than 150µm in length were observed from 64.2% of neurons examined. Insufficient protein concentration was present in the non-bound elutant to determine whether the non heparin binding fraction of cortical astroglial CM was biologically active.

DISCUSSION

The results of the preliminary biochemical analysis reported in this study indicate that astroglia may mediate dendrite growth from cortical neurons in

culture by a soluble, heparin binding protein with a molecular weight between 10 and 100kDa. Fibroblast growth factor (FGF), a heparin binding peptide growth factor was observed to support similar dendrite growth. Axon growth, however, was also supported by FGF, which does not fully explain the regional differences that astroglial demonstrate in promoting dendrite, but not axon growth. In addition, protein extraction and gel electrophoresis studies suggest more than one soluble, astroglial released factor may be capable of mediating dendrite growth.

Astroglial conditioned medium and dendrite growth.

Previous studies have provided evidence that astroglial conditioned medium can mediate dendrite growth from mesencephalic (Rousselet et al 1988 and 1990) or cerebellar neurons (Seil et al 1992) *in vitro*. The extent to which dendrite growth is supported, however, is not clear. For example, astroglial conditioned media has been observed to promote Purkinje cell dendritic spine growth in some studies (Seil et al 1992), whereas other investigations suggest that astroglia provide some, but not all the necessary signals for complete dendritic maturation of Purkinje cells (Baptista et al 1994).

It is not known how diffusible factors released by astroglia mediate dendrite growth. Prochiantz and colleagues have postulated that astrocyte derived factors capable of regulating dendrite growth primarily modulate adhesion of the neuron to the substrate (Prochiantz et al 1990, Rousselet et al 1990, LaFont et al 1992). Promotion of adhesion by diffusible factors, however, appears to depend on the presence of extracellular matrix (ECM) components. For example, FGF promotes the adherence of embryonic chick neural tube cells to ECM components but not to polycationic substances such as polylysine

(Kinoshita et al 1993). When astrocytes are cultured without serum, as in our coculture experiments, ECM components are frequently absent (Ard et al 1991). In addition, when we examined the role of astroglial diffusible factors, neurons were cultured on polylysine yet the influence of astroglial conditioned medium on process outgrowth was still observed. It is thus feasible that diffusible factors stimulate dendrite growth directly through an as yet unidentified second messenger signaling pathway and in turn greater dendrite growth contributes to increased cell adhesion.

Our results and those of others (Rousselet et al 1988 and 1990) are, in part, consistent with the hypothesis that a diffusible factor or factors released by astroglia are responsible for the regional heterogeneity observed in astroglial mediated dendrite growth. Other co-culture experiments, however, do not support this model. For example, Qian et al (1992) observed that axon, and not dendrite growth from spinal cord and hippocampal neurons was differentially supported by medium conditioned by astrocytes derived from different CNS regions; in these experiments a noncontact coculture system, rather than conditioned medium was used.

The biochemical characteristics of astroglial conditioned medium associated with dendrite growth have not been extensively investigated. Protein degradation studies, however, suggest that a protein or protein cofactor present in astroglial conditioned medium can mediate dendrite growth (Rousselet et al 1988, Seil 1992). Although the precise molecular weight of this factor is not known, ultrafiltration and fractionation of astroglial conditioned medium indicate it has a molecular weight greater than 10kDa (Matthiessen et al 1989, Seil 1992).

Extracellular matrix molecules are found in astroglial conditioned medium that supports dendrite growth. For example, medium conditioned by mesencephalic astrocytes supports dendritic arborization from mesencephalic neurons *in vitro*; laminin, fibronectin and proteoglycans can be found in immunoblots of the medium (Rousselet et al 1988 and 1990). Quantitative immunoprecipitation experiments, however, demonstrate that medium conditioned by striatal astrocytes, which does not support dendrite growth from mesencephalic neurons, contains similar quantities of these matrix molecules. When separated into soluble and insoluble fractions by ultracentrifugation, the dendrite promoting properties of mesencephalic astrocyte conditioned medium are retained in the soluble fraction even though the same extracellular matrix components are found in both fractions (Rousselet et al 1990). The differential morphogenetic effects and regional heterogeneity that astroglial conditioned medium demonstrates may thus be attributable to different isoforms of the same molecule.

Our preliminary biochemical analysis of cortical astroglial conditioned medium that supports dendrite growth, suggests that a heparin binding factor may be required for dendrite growth. The electrophoretic profile of other astroglial conditioned medium known to support dendrite elaboration, however, suggests that more than one factor may promote dendrite growth. Two factors known to promote neurite growth *in vitro*, fibroblast growth factor (Morrison et al 1986, Walicke et al 1986, Hatten et al 1988, Mattson et al 1989) and fibronectin (Carbonetto et al 1983) are distinguished by heparin binding (Shing et al 1984, Price and Hynes 1985, Burgess and Maciag 1989, Thierry and Boyer 1992). The role of these molecules and other potential

molecular mechanisms of astroglial mediated dendrite growth including: cell adhesion molecules, other peptide growth factors, and components of the extracellular matrix is reviewed in the remainder of the chapter.

Peptide growth factors and dendrite growth.

Polypeptide growth factors mediate a variety of cell-cell interactions and are capable of promoting neuron proliferation, migration, differentiation, survival, and neurite growth in the CNS (Walicke et al 1986, Barde 1989, Reynolds and Weiss 1992, Svendsen et al 1994). The prototypical growth factor is nerve growth factor which was initially demonstrated to be required for neuronal survival in the peripheral and sympathetic nervous systems (Levi-Montalcini 1987). In addition, NGF can induce dendritic arborization from sympathetic neurons *in vivo* (Snider 1988) or vertebrate sensory neurons *in vitro* (De Koninck et al 1993).

In the CNS nerve growth factor has a more limited role (Hefti et al 1984, Kromer et al 1987, Barde 1989, Cattaneo and McKay 1990, Svendsen et al 1994) and has not been observed to promote neurite growth from CNS neurons (Banker 1980, Morrison et al 1987, Mattson and Rychlik 1990). Indirect evidence, however, suggests that homologous members of the neurotrophin family, brain derived growth factor (BDNF) and neurotrophin 3 (NT-3, Barde 1989), may be capable of mediating dendrite growth in the cerebellum. For example, in primary cultures of Purkinje cells, granule cells regulate neuron differentiation and maturation of dendrites (Baptista et al 1994). These same cells express the neurotrophins NT-3 and BDNF (Lindholm et al 1993).

Can astroglia release neurotrophins or other peptide growth factors that can mediate dendrite growth? As discussed in chapter 9 immunohistochemical and *in situ* hybridization studies have demonstrated that astroglia can express a variety of growth factors or their mRNA *in vitro* and *in vivo*. Some of these factors such as TGF (Chalazonitis et al 1992), IGF-1 (Ang et al 1993), EGF (Morrison et al 1987) and FGF (Morrison et al 1986, Walicke et al 1986, Hatten et al 1988) have been observed to promote neurite growth from peripheral, spinal cord or central neurons; a specific promotion of dendrite growth from cortical neurons, however, has not been reported.

Fibroblast growth factor.

Several peptide growth factors were tested in this study; only acidic and basic fibroblast growth factor were observed to promote primary dendrite extension similar to that observed when cortical neurons were grown in medium conditioned by cortical astroglia. Both aFGF and bFGF are found in relatively high levels in the CNS (Burgess and Maciag 1989); immunohistochemical and biochemical studies demonstrate that FGF and its mRNA is primarily localized in astrocytes (Ferrara et 1988, Emoto et al 1989, Gomez -Pinilla et al 1992, Woodward et al 1992). Whether FGF is found in medium conditioned by astrocytes, however, is not clear (Hatten 1988, Woodward et al 1992). Our preliminary results suggest biologically active FGF may be present in astroglial conditioned medium. FGF has a molecular weight of 18kDa and is distinguished by strong heparin binding (Shing et al 1984, Burgess and Maciag 1989); these biochemical characteristics are consistent with the dendrite promoting properties demonstrated by biochemical analysis of medium conditioned by cortical astroglia.

A variety of potent *in vitro* effects for FGF have been identified in the developing and adult central nervous system (Cheng and Mattson 1991, Pittack et al 1991, Yoshida and Gage 1991, Engele and Bohn 1992, Chernusek 1993, Maiese et al 1993, Meiners et al 1993, Vescovi et al 1993). In particular, tissue culture experiments demonstrate that the addition of FGF to culture medium improves survival and increases the number of process bearing neurons derived from several different CNS regions including cerebellum (Hatten et al 1988), cerebral cortex (Morrison et al 1986), and hippocampus (Walicke et al 1986, Mattson et al 1989).

Can FGF promote dendrite growth? A specific effect on dendrite growth was not reported in the studies described above (Morrison et al 1986, Walicke et al 1986, Hatten et al 1988). We observed that the addition of FGF to the culture medium supported primary dendrite growth from cortical neurons. The effect, however, was not specific for dendrites since axon growth was also promoted. These findings are, in part, consistent with previous studies in which the effects of astroglial conditioned medium and FGF on mesencephalic neurons have been compared; whereas FGF supported dendrite growth, the extent of FGF mediated arborization was not as great as that observed in conditioned medium (Rousselet et al 1988). Studies in other systems, such as the sympathetic nervous system, did not find that FGF promoted dendrite elaboration (Lein and Higgins 1989); collectively these observations suggest that the specific effect of FGF on dendrite growth is far from established.

It is possible that FGF, alone, provides a non-specific promotion of neurite growth through its neurotrophic effect (Walicke et al 1986, Mattson et al

1989). For example, when added to hippocampal neurons *in vitro* FGF decreases glutamate neurotoxicity, primarily by reducing the expression of NMDA receptor proteins (Mattson et al 1993). This decreased vulnerability to neurotoxicity is associated with FGF attenuation of glutamate mediated dendrite regression (Mattson et al 1989). Specific effects of FGF, such as selective modulation of neurite outgrowth (Walicke et al 1986, Uniscker et al 1987) may only result from interactions with the cell surface and the extracellular matrix (Gospodarowicz 1991, Rusolahti and Yamaguchi 1991, Yayon et al 1991, Letourneau et al 1994).

The extracellular matrix, proteoglycans and dendrite growth.

The extracellular matrix includes many diverse secreted molecules that are immobilized outside cells (Rutka et al 1988, Sanes 1989, Reichardt and Tomaselli 1991, Letourneau et al 1994). Several extracellular matrix components such as tenascin (Kruse et al 1985, Steindler et al 1989, Crossin et al 1990), laminin (Liesi et al 1983, Price and Hynes 1985, Ard and Bunge 1988, Matthiessen et al 1989), fibronectin (Price and Hynes 1985, Liesi et al 1986, Matthiessen et al 1989), and proteoglycans (Ard and Bunge 1988, Johnson-Green et al 1992, LaFont et al 1992) are expressed by astrocytes during CNS development.

Immunohistochemical and *in situ* hybridization studies *in vivo* demonstrate that ECM components have acquired specialized functions and are generally expressed in the CNS during neuron migration, axon growth or compartmentation of the CNS (Dodd and Jessel 1988, Sanes 1989, Snow et al 1990a and 1990b, Steindler 1993, Letourneau et al 1994). Several *in vitro* experiments, however, suggest that some soluble ECM components, when

added to the culture medium, may also mediate neuron morphogenesis and dendrite growth (Chamak and Prochiantz 1989, Lein and Higgins 1989, LaFont et al 1992). By contrast, neurons grown on tenascin, laminin, or fibronectin culture substrates do not form mature dendritic arbors, instead axon elongation is favored (Lochter et al 1993, Baptista et al 1994). For example, when embryonic mesencephalic neurons are grown on a fibronectin substrate the majority of cells are unipolar and extend only an axon, whereas when soluble fibronectin is added to the culture medium the majority of neurons are multipolar (Chamak and Prochiantz 1989).

Indirect evidence suggests soluble fibronectin may be responsible for dendrite growth from other neuronal populations. For example, soluble, but not bound, basement membrane extract derived from EHS sarcoma (BME) promotes dendrite growth from cultured sympathetic neurons (Lein and Higgins 1989). BME is similar to the high molecular weight fraction of serum that also promotes dendrite growth from cultured sympathetic neurons (Bruckenstein and Higgins 1988). Fibronectin and thrombospondin are common to both BME and serum. Can fibronectin promote cortical neuron dendrite growth? Astroglia in culture synthesize and secrete fibronectin into the culture medium that binds to heparin (Price and Hynes 1985, Thierry and Boyer 1992) consistent with our observation that a heparin binding protein found in astroglial conditioned medium supports dendrite growth. Fibronectin, however, is found enriched in high molecular weight subunits. By contrast, we did not observe dendrite growth in the astroglial conditioned fraction greater than 100kDa, suggesting that fibronectin may not be responsible for cortical neuron dendrite growth.

Another important component of the extracellular matrix, proteoglycans may be involved in dendrogenesis. Proteoglycans are molecules composed of a protein core that carry long chains of various sulfated sugars or glycosaminoglycans (GAGs); dermatan, heparan and chondroitin sulfate are most commonly found in the brain (Herndon and Landon 1990, Letourneau et al 1994). Astrocytes express proteoglycans on their surface and excrete them into the extracellular environment (Johnson-Green et al 1992, LaFont et al 1992). Whereas proteoglycans have generally been observed to direct axon growth and not promote neurite growth (Carbonetto et al 1983, Snow et al 1990a and 1990b, Brittis et al 1992), specific glycosaminoglycans appear to influence neuron polarity. For example, when added as soluble molecules GAGs, such as heparan and chondroitin sulfate favor axon elongation and fasciculation, whereas dermatan sulfate supports both dendrite and axon growth from hippocampal neurons in culture (LaFont et al 1992). Substrate bound GAGs, however, do not promote neurite growth (Carbonetto et al 1983, Snow et al 1990a and 1990b, LaFont et al 1992).

Proteoglycans and other components of the extracellular matrix bind many soluble substances including growth factors (Gospodarowicz 1991, Rusolahti and Yamaguchi 1991, Thierry and Boyer 1992). For example, the mode of action of FGF is intimately linked to the extracellular matrix (Gospodarowicz et al 1986, Yayon et al 1991). There are several interactions between the ECM and soluble factors that may be of potential benefit to dendrite growth: 1) the local trophic effect of the soluble factor may be augmented by protecting it from degradation and providing a matrix bound reservoir (Yayon et al 1991), 2) protein kinases or other second messenger systems that can alter cytoskeletal composition can be activated (Bixby 1989, Yayon et al 1991), 3) a

direct link between the soluble factor and cytoskeleton can be provided (Pollerberg et al 1986, Rapraeger et al 1986), and 4) internalization and intracellular targeting of growth factors to cytoplasmic or nuclear signal transducing receptors can occur (Rusolahti and Yamaguchi 1991, Damsky and Werb 1992, Thierry and Boyer 1992, Woodward et al 1992). Collectively, these interactions may be fundamental to translating a soluble factor released by astroglia into a regionally specific, instructive signal for dendrite growth.

Cell adhesion and dendrite growth.

Cell adhesion has been implicated in cell-cell sorting, cell migration, axon growth, and the formation of neuronal connections during development of the nervous system (Dodd and Jessel 1988, Grumet 1991, Doherty and Walsh 1992); does adhesion influence dendrite growth? Several experimental observations suggest that dendrite growth *in vitro* may require high adhesion conditions, whereas axons grow in low adhesion conditions (Prochiantz et al 1990, Rousselet 1990, LaFont et al 1993, Lochter et al 1993). First, the correlation of a small cell body diameter and absence of dendrites *in vitro* has been observed in sympathetic (Bruckenstein and Higgins 1988) and central neurons (Autillo-Touati et al 1988, Rousselet et al 1990), whereas cells that bear dendrites exhibit a larger cell body surface (Rousselet et al 1990, Lochter et al 1993). Second, neurons grown under conditions permissive for dendrite growth are less likely to detach from the culture substrate after shaking than neurons that do not elaborate dendrites (Lieth et al 1990, Rousselet et al 1990). Finally, cytoskeletal perturbation experiments demonstrate that decreasing the compression force exerted by microtubules to overcome low adhesion conditions affects axon growth to a greater extent than dendrite growth (Abosch and Lagenauer 1993, LaFont et al 1993).

The relationship between adhesion and dendrite growth may not be straightforward. First, the model proposed by Prochiantz and colleagues suggests that axon growth is initiated in low adhesion conditions whereas dendrite growth is initiated in high adhesion conditions (Prochiantz et al 1990). The implication of this model, that a neurite is differentiated as soon as it is initiated, is not consistent with: 1) observations of developing neurites in culture made by Banker and colleagues (Cáceres et al 1986, Dotti et al 1988, Goslin et al 1990, Kleinman et al 1994), or observations described in chapter 8 which demonstrate that neurites are not differentiated at initiation of outgrowth, and 2) observations described in chapter 10 that demonstrate that astroglia are primarily responsible for the maintenance of the dendritic arbor rather than the initiation of process outgrowth. Second, the association between cell adhesion and cell spreading is not clear. For example, measurements of the adhesive forces developing between cells, or between cells and substrate demonstrate dissociation between the initial binding and subsequent cell spreading of fibroblasts (Lotz et al 1989). We did not observe an association between cell body diameter and dendrite growth (see figure 11b). Adhesion, however, was not quantified. These results, although not conclusive, suggest that there may not be a simple relationship between dendrite growth, cell spreading and adhesion. Finally, it is not clear from the experiments described by Prochiantz and colleagues (Rousselet et al 1990, LaFont et al 1992) and other investigators (Leith et al 1990, Lochter et al 1993) what comes first: does an extensive dendritic arbor result from increased adhesion or is increased adhesion a consequence of greater dendritic growth?

Other experiments suggest that a simple functional correlate between the degree of adhesion and neurite growth may not exist (Buettner and Pittman 1991, Kapfhammer and Schwab 1992, Lemmon et al 1992, Smith 1994). For example, antibodies to N-cadherin or integrin B1 inhibit neurite elongation from retinal neurons growing on astroglial monolayers, but quantitative attachment assays do not demonstrate an association between this inhibition and decreased neuronal adhesion to the astroglial monolayers (Neugebauer et al 1988). Instead adhesive signals from the environment may lead to changes in cytoplasmic second messengers such as calcium, inositol phosphates, or protein kinases that in turn can regulate cytoskeletal organization and provide an instructive signal for process growth (Bixby 1989, Reichardt and Tomaselli 1991, Atashi et al 1992, Damsky and Werb 1992, Thierry and Boyer 1992, Lee 1993).

A large variety of different molecules, collectively known as cell adhesion molecules (CAM's) mediate adhesion. The cell adhesion molecules include three families of integral membrane proteins: 1) the immunoglobulin superfamily, 2) the cadherin family, and 3) integrins (Edelman 1987, Takeichi 1988, Crossin et al 1990, Grumet 1991, Doherty and Walsh 1992). Neurite outgrowth on astroglial surfaces has been demonstrated to be dependent on several adhesion molecules (Neugebauer et al 1988, Tomaselli et al 1988); whether these molecules can specifically mediate dendrite growth is not known.

Growth factors can regulate cell adhesion and in turn CAM's can modify the cellular response to growth factors (Saad et al 1991, Thierry and Boyer 1992, Kinoshita et al 1993). The interaction between growth factors and CAM's can

occur in both a paracrine and autocrine fashion and may, in part, be responsible for translating the release of diffusible factors into locally relevant cues for dendrite growth. In addition, several aspects of CAM structure, such as multiple exon genes, multiple structural domains that exhibit different biological functions, and a high degree of glycosylation (Grumet 1991, Reichardt and Tomaselli 1991, Frei et al 1992, Kapfhammer and Schwab 1992, Appel et al 1993) may give rise to regional or developmental specific isoforms through alternative RNA splicing or post-translational processing (Pollerberg et al 1987, Doherty et al 1990, Acheson et al 1991, Doherty and Walsh 1992, Mayford et al 1992, Zhang et al 1992). The functional significance of these structural attributes are not known; it is plausible, however, that they may contribute to the regional heterogeneity observed in astroglial mediated dendrite growth.

Molecular diversity and synergism.

How do regional differences in astroglial mediated dendrite growth occur at a molecular level? The structure of various molecules and their receptors that may be involved in dendrite growth encodes a tremendous potential functional diversity (Reichardt and Tomaselli 1991, Damsky and Werb 1992, Doherty and Walsh 1992, Frei et al 1992, Hynes et al 1992, Appel et al 1993, Tomaselli et al 1993, Letourneau et al 1994). Multiple genes, alternative splicing and distribution of the various receptors further increase the functional diversity of these molecules (Acheson et al 1991, Reichardt and Tomaselli 1991, Hunter et al 1991, Zhang et al 1992). Astrocytes in different regions, therefore, may release: 1) unique molecules for that region, 2) different concentrations of the same molecule, 3) molecules at different times, or 4) different isoforms of the same molecule.

Rather than molecular structural heterogeneity, regional heterogeneity in astroglial mediated dendrite growth may also result from cellular characteristics of individual neurons or astroglia complementing the action of one or two astroglial diffusable factors. First, soluble factors such as peptide growth factors can influence ECM production or regulate receptor expression and adhesion strength (Rossino et al 1990, Thierry and Boyer 1992, Kinoshita et al 1993, Meiners et al 1993). In turn cell-cell and cell-substrate adhesion molecules modify the cellular response to growth factors (Saad et al 1991, Thierry and Boyer 1992, Kinoshita et al 1993). Second, growth factors and cytokines regulate the expression of other growth factors (Huff and Schreier 1990, Yoshida and Gage 1991, Zafra et al 1992, Chernusek 1993), or are biologically active only when combined with other soluble peptide factors (Cattaneo and McKay 1990, Vescovi et al 1993). Third, neurotransmitters and neural activity appear to work in concert with some growth factors to determine neuron development, (Mattson et al 1989, Cohen-Cory et al 1991). Finally, the different external signals from growth factors, extracellular matrix, neurotransmitters, and cell adhesion molecules can converge on the same cellular pathways and cooperate to induce a biological response (Damsky and Werb 1992, Thierry and Boyer 1992, Letourneau et al 1994, Williams et al 1994). The signal transduction mechanisms that connect intercellular interactions with intracellular control of process growth, however, are just beginning to be understood.

The molecular mechanisms that mediate dendrite growth are far from fully elucidated but appear complex and diverse. Furthermore, it is becoming increasingly apparent that molecular mechanisms such as peptide growth

factors, components of the extracellular matrix and cell adhesion molecules are part of a complex interactive process that mediates neuron differentiation, maturation and function (Cattaneo and McKay 1990, Saad et al 1991, Damsky and Werb 1992, Doherty and Walsh 1992, Thierry and Boyer 1992, Kinoshita et al 1993, Meiners et al 1993, Letourneau et al 1994). Part of the challenge of developmental neurobiology is to unravel the molecular mechanisms of dendrite growth. The preliminary results described in this report are far from the answer, but provide needed initial information.

CONCLUDING REMARKS

The vast majority of neurons in the central nervous system extend two types of processes, axons and dendrites. The precise mechanisms that regulate growth of dendrites remain largely unknown, however, an understanding of dendrite growth is important for several reasons: 1) dendrite structure underlies many aspects of normal CNS function (Stewart 1989, Midtgaard 1994) and adaptation to the environment, 2) dendrite growth appears to compensate for neuronal loss in the aging CNS (Coleman and Flood 1986) and correlate with functional recovery after injury (Jones and Schallert 1994), and 3) dendritic abnormalities are observed in many acute neurologic insults (Mattson et al 1988a, Kitigawa et al 1989, Taft et al 1992, Shigeno et al 1993) and chronic neurodegenerative conditions (Buell and Coleman 1979, McKee et al 1989, Jacobsen 1991).

Numerous factors, that influence or may influence growth and maturation of the dendritic arbor of central nervous system neurons have been identified (summarized in Table 10). The results of the experiments reported in this study are consistent with and extend previous observations that astroglia can influence the shape of the dendritic arbor elaborated by CNS neurons (Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988, Rousselet et al 1988, Prochiantz et al 1990, Rousselet et al 1990). The novel and important findings of this study are:

- 1) Astroglia can influence dendrite growth from cerebral cortical neurons.

- 2) The astroglial influence on dendrite growth is principally on primary dendrite number rather than on the length of dendrites.
- 3) Astroglia demonstrate regional differences in their ability to maintain, but not initiate, cortical neuron primary dendrite growth.
- 4) Homotypic astroglia appear best able to maintain the primary dendritic arbor once it is elaborated; the ability to support primary dendrite growth, however, is not confined to homotypic astroglia only.
- 5) Axon and dendrite growth are separately regulated by astroglia, however, the number of primary dendrites maintained by cortical neurons *in vitro* may be related to the cell's axon growth.
- 6) Mature astroglia are able to support primary dendrite, but not axon growth.
- 7) Reactive astroglia are able to support primary dendrite but not axon growth.
- 8) Astroglia release a diffusible factor or factors that can specifically influence the growth of primary dendrites, either alone or in combination with other factors.
- 9) Diffusible heparin binding proteins that have a molecular weight between 10 and 100kDa may play a role in astroglial mediated dendrite growth.

TABLE 10: FACTORS AFFECTING THE DEVELOPMENT OF THE DENDRITIC ARBOR OF NEURONS IN THE CENTRAL NERVOUS SYSTEM

| FACTOR | REFERENCE |
|-----------------------------------|---|
| Intrinsic | Van der Loos 1965, Bartlett and Banker 1984a, Banker and Waxman 1988, Neale et al 1993 |
| Cytoskeleton | Matus et al 1986, Matus 1988 |
| Neuron density | Chamak et al 1987 |
| Afferent innervation | Rakic and Sidman 1971, Cowan et al 1980, Rubel et al 1981, Perry and Linden 1982, Deitch and Rubel 1984, Mattson et al 1988, Leventhal et al 1989 |
| Synapse formation | Vaughan 1989, Schilling et al 1991 |
| Neurotransmitters | Mattson et al 1988a, 1989b |
| Neuronal activity | Weiss and Pysh 1978, Smith 1981, Rogowoski and Feng 1982, Greenough et al 1985, Ramoa et al 1988, Sanes et al 1992a and 1992b, Jones and Schallert 1994 |
| Sensory deprivation or enrichment | Valverde 1968, Globus et al 1973 |
| Local astroglial environment | Denis-Donini et al 1984, Chamak et al 1987, Autillo-Touati et al 1988 |
| Astroglial diffusible factors | Rousselet et al 1988 and 1990, Seil et al 1992 |
| Peptide growth factors | Mattson et al 1989 |
| Extracellular matrix components | Chamak and Prochiantz 1989 |
| Proteoglycans/glycosaminoglycans | LaFont et al 1992 |

| | |
|-----------|--|
| Adhesion | Prochiantz et al 1990, Rousselet et al 1990, LaFont et al 1993 |
| Hormones | Goldstein et al 1990, McEwan 1991 |
| Opioids | Hauser et al 1989 |
| Nutrition | Rees and Harding 1988 |

Table 10: The table summarizes select published data describing factors that influence or are postulated to influence the morphology of the dendritic arbor elaborated by neurons in the central nervous system.

Do these tissue culture observations correlate with *in situ* or *in vivo* findings? Transplant and lineage studies of the developing cortex (Renfranz et al 1991, Schlagger and O'Leary 1991, McConnel 1992), and a variety of studies using Golgi, autoradiographic, ultrastructural, biochemical and immunohistochemical techniques *in situ* indicate that cortical neuron dendrite growth occurs once neurons have reached their final position in the developing mammalian cortex (Ramon y Cajal 1954, Shoumakis and Hinds 1978, Crandall et al 1986, Miller 1988). Furthermore, qualitative and quantitative *in situ* studies in many areas of the developing CNS, including the cerebral cortex, demonstrate that primary dendritic projections, although immature are established before the arrival of afferents and formation of synapses (Wise et al 1979, Hammer et al 1981, Petit et al 1988, Snider et al 1992, Sutton and Brunso-Bechtold 1993). Subsequent dendritic development, maturation, and refinement is influenced by afferents and synaptic activity (Weiss and Pysh 1978, Smith 1981, Rogowoski and Feng 1982, Greenough et al 1985, Ramoa et al 1988, Leventhal et al 1989, Black et al 1990, Kolb and Gibb 1991, Sanes et al 1990, 1992a, and 1992b, Jones and Schallert 1994). Thus, *in vivo*, it is likely that the local astroglial environment can influence maintenance of the primary dendritic arbor.

We propose therefore that: 1) neurons have an intrinsic ability to initiate growth of multiple undifferentiated dendrites, 2) the basic structure of the neuron, the primary dendritic arbor, is largely determined by activity independent mechanisms and maintained by the local astroglial environment, and 3) establishment of the fully differentiated dendritic arbor and its subsequent refinement into a highly tuned and functional circuit with other neurons depends on neuronal activity and environmental influences.

These postulates are consistent with a recent study which demonstrated that a combination of purified cerebellar astroglia and granule cells mediated full differentiation of Purkinje cell dendrites in culture (Baptista et al 1994).

What is the biological significance of the results reported in this study? A mechanism allowing separate but coordinated regulation of axonal and dendritic growth may represent a critical factor in the establishment of neuronal circuitry. For example, the repression of dendrite growth may reduce the time for the axon to reach its specific target, particularly when the target is a source of further neurotrophic factors. Alternatively, the basic form of a neuron, its primary neuritic arbor, prior to the arrival of afferents may be critical for establishing appropriate and specific connections in the CNS. In the mature CNS the maintenance of the primary dendritic arbor and coordinated dendrite and axon growth may represent a means to adapt to the ever changing environment, whereas in the injured CNS these mechanisms may prove crucial in reforming appropriate neuronal connections and restoring function. The preliminary biochemical findings in this study suggest that it may eventually be feasible to develop an endogenous means of recreating these events.

Astroglia are but one of many environmental signals which, in conjunction with intrinsic factors, influence the final form and function of neurons in the central nervous system (Table 10). It is unlikely that astroglia act in isolation or promote dendrite growth in a simple fashion. Instead intrinsic and extrinsic signals interact and act in cumulative and diverse ways to regulate dendrite development and differentiation. The challenge of developmental neurobiology is to determine how the immense number of signals are

coordinated and information interpreted to generate the enormous variety of dendritic arbors and their complex interrelationships in the CNS. An understanding of these developmental processes has implications in such pathologic conditions as head and spinal cord injury, stroke, subarachnoid hemorrhage and degenerative conditions such as Parkinson's and Alzheimer's Disease. While these results may not be immediately transferable to the clinical treatment of neurologic disorders, they are essential if we are ever to devise systematic treatment to actively promote recovery and regeneration of the injured or diseased brain.

LITERATURE CITED

- Abosch A, Lagenaur C (1993): Sensitivity of neurite outgrowth to microfilament disruption varies with adhesion molecule substrate. *J Neurobiol* 24:344-355.
- Acheson A, Sunshine JL, Rutishauser U (1991): NCAM Polysialic acid can regulate both cell-cell and cell substrate interactions. *J Cell Biol* 114:143-153.
- Ahmed Z, Lewis CA, Faber DS (1990): Glutamate stimulates release of Ca^{2+} from internal stores in astroglia. *Brain Res* 516:165-169.
- American Heart Association (1986): Stroke Facts, Dallas, American Heart Association.
- Anders JJ, Johnson JA (1990): Transection of the rat olfactory nerve increase glial fibrillary acid protein immunoreactivity from the olfactory bulb to the piriform cortex. *Glia* 3:17-25.
- Anezaki T, Yamagisawa K, Takahashi H, Nakajima T, Miyashita K, Ishikawa A, Ikuta F, Miyatake T (1992): Remote astrocytic response of prefrontal cortex is caused by lesions in the nucleus basalis of Meynert but not in the ventral tegmental area. *Brain Res* 574:63-69.
- Ang LC, Bhaumick B, Juurlink BHJ (1993): Neurite promoting activity of insulin, insulin-like growth factor I and nerve growth factor on spinal motoneurons is astrocyte dependent. *Dev Brain Res* 74:83-88.
- Aoki E, Semba R, Mikoshiba K, Kashiwamata S (1991): Predominant localization in glial cells of free L-arginine: immunocytochemical evidence. *Brain Res* 547:190-192.
- Appel F, Holm J, Conscience JF, Schachner M (1993): Several extracellular domains of the neural adhesion molecule L1 are involved in neurite outgrowth and cell body adhesion. *J Neurosci* 13:4764-4775.
- Ard MD, Bunge M, Wood PM, Schachner M, Bunge RP (1991): Retinal neurite growth on astrocytes is not modified by extracellular matrix, anti-L1 antibody or oligodendrocytes. *Glia* 4:7-82.
- Ard MD, Bunge RP (1988): Heparan sulfate proteoglycan and laminin immunoreactivity on cultured astrocytes: relationship to differentiation and neurite growth. *J Neurosci* 8:2844-2858.
- Atashi JR, Klinz SG, Ingraham CA, Matten WT, Schachner M, Maness P F (1992): Neural cell adhesion molecules modulate tyrosine phosphorylation of tubulin in nerve growth cone membranes. *Neuron* 8:831-842.
- Autillo-Touati A, Chamak B, Araud D, Vuillet J, Seite R, Prochiantz A (1988): Region-specific neuro-astroglial interactions: ultrastructural study of the *in vitro* expression of neuronal polarity. *J Neurosci Res* 19:326-342.
- Awgulewitsch A, Jacobs D (1990): Differential expression of Hox 3.1 protein in subregions of the embryonic and adult spinal cord. *Development* 108:411-420.
- Baas PW, Black MM, Banker GA (1989): Changes in microtubule polarity orientation during the development of hippocampal neurons in culture. *J Cell Biol*, 109:3085-3094

- Baas PW, Deitch JS, Black MM, Banker GA (1988): Polarity orientation of microtubules in hippocampal neurons: Uniformity in the axon and nonuniformity in the dendrite. *Proc Natl Acad Sci USA* 85:8335-8339.
- Baas PW, White LA, Heidemann SR (1987): Microtubule polarity reversal accompanies regrowth of amputated neurites. *Proc. Natl. Acad. Sci USA*. 84:5272-5276
- Baird DH, Hatten ME, Mason CA (1992): Cerebellar target neurons provide a stop signal for afferent neurite extension *in vitro*. *J Neurosci* 12:619-634.
- Baker AJ, Moulton RJ, MacMillan VH, Shedden PM (1993): Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans. *J Neurosurg* 79:369-372.
- Bakhit C, Armanini M, Bennett GL, Wong WLT, Hansen SE, Taylor R (1991): Increase in glia derived nerve growth factor following destruction of hippocampal neurons. *Brain Res* 560:76-83.
- Balasingam V, Tejada-Berges T, Bouckova R, Yong VW (1994): Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines *J Neurosci* 14:846-856.
- Bamburg J R, Bray D, Chapman K (1986): Assembly of microtubules at the tip of growing axons. *Nature* 321:788-790.
- Banker GA (1980): Trophic interactions between astroglial cells and hippocampal neurons in culture. *Science* 209:809-810.
- Banker GA, Waxman AB (1988): Hippocampal neurons generate natural shapes in cell culture. In Lasek FJ, Black MM (eds): "Neurology and Neurobiology. Vol. 37, Intrinsic Determinants of Neuronal Form and Function." New York: Alan R. Liss, pp 61-82.
- Baptista CA, Hatten ME, Blazeksi R, Mason CA (1994): Cell-cell interactions influence survival and differentiation of purified Purkinje cells *in vitro* *Neuron* 12:243-260.
- Barbin G, Katz DM, Chamak B, Glowinski J, Prochiantz A (1988): Brain astrocytes express region-specific surface glycoproteins in culture. *Glia* 1:96-103.
- Barde YA (1989): Trophic factors and neuronal survival. *Neuron* 2:1525-1534.
- Barres BA (1991): New roles for glia. *J Neurosci* 11:3685-3694.
- Barres BA, Koroshetz WJ, Chun LLY, Corey DP (1990a): Ion channel expression by white matter glia: the type 1 astrocyte. *Neuron* 5:527-544.
- Barres BA, Koroshetz WJ, Swartz KJ, Chun LLY, Corey DP (1990b): Ion channel expression by white matter glia: the O2A glial progenitor cell. *Neuron* 5:507-524.
- Barrett CP, Donati EJ, Guth L (1984): Differences between adult and neonatal rats in their astroglial response to spinal injury. *Exp Neurol* 84:374-385.
- Bartlett WP, Banker GA (1984a): An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture I. Cells which develop without intercellular contacts. *J Neurosci* 4:1944-53.

- Bartlett WP, Banker GA (1984b): An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture II. Synaptic relationships. *J Neurosci* 4:1954-1965.
- Bastmeyer M, Bähr M, Stuermer CAO (1993): Fish optic nerve oligodendrocytes support axonal regeneration of fish and mammalian retinal ganglion cells. *Glia* 8:1-11.
- Beal MF (1992): Role of excitotoxicity in human neurological disease. *Curr Opin Neurobiol* 2:657-662.
- Behar T, McMorris FA, Novotny EA, Barker JL, Dubois-Dalcq M (1988): Growth and differentiation properties of O-2A progenitors purified from rat cerebral hemispheres. *J Neurosci Res* 21:168-180.
- Beltz BS, Burd GD (1989): Immunocytochemical techniques. Principles and Practice. Blackwell Scientific Publications, Cambridge, MA.
- Benevise EN, Sparacio SM, Norris JG, Grenett HE, Fuller GM (1990): Induction and regulation of interleukin-6 gene expression in rat astrocytes. *J Neuroimmunol* 30:210-212.
- Berry M, Maxwell WL, Logan A, Mathewson A, McConnell P, Ashhurst DE, Thomas GH (1983): Deposition of scar tissue in the central nervous system. *Acta Neurochir (suppl)* 32:31-53.
- Beull SJ, Coleman PD (1979): Dendritic growth in the aged human brain and failure of growth in senile dementia. *Science* 206:854-856.
- Bevan S (1990): Ion channels and neurotransmitter receptors in glia. *Semin Neurosci* 2:467-481.
- Binder LI, Frankfurter A, Kim H, Cáceres A, Payne M, Rebhun LI (1984): Heterogeneity of microtubule-associated protein 2 during rat brain development. *Proc Natl Acad Sci USA* 81:5613-5617.
- Bixby JL (1989): Protein kinase C is involved in laminin stimulation of neurite outgrowth. *Neuron* 3:287-297.
- Bixby JL, Harris WA (1991): Molecular mechanisms of axon growth and guidance. *Annu Rev Cell Biol* 7: 117-159.
- Björklund A, Stenevei U (1984): Intracerebral neural implants: Neuronal replacement and reconstruction of damaged circuitries. *Ann Rev Neurosci* 7:279-308.
- Black JA, Friedman B, Waxman SG, Elmer LW, Angelides KJ (1989): Immuno-ultrastructural localization of sodium channels at nodes of Ranvier and perinodal astrocytes in rat optic nerve. *Proc R Soc Lond [Biol]* 238:39-51.
- Black JA, Sontheimer J, Waxman SG (1993): Spinal cord astrocytes *in vitro*: phenotypic diversity and sodium channel immunoreactivity. *Glia* 7:272-285.
- Black JE, Isaacs KR, Anderson BJ, Alcantara AA, Greenough WT (1990): Learning causes synaptogenesis whereas motor activity causes angiogenesis in cerebellar cortex of adult rats. *Proc Natl Acad Sci USA* 87:5568-5572.
- Blakemore WF, Franklin RJ (1991): Transplantation of glial cells into the CNS. *TINS* 14:323-327.

- Blessed G, Tomlinson BE, Roth M (1968): The association between quantitative measures of dementia and of senile change in the cerebral grey matter of elderly subjects. *Br J Psychiat* 114:797-811.
- Bloch-Gallego E, Le Roux I, Joliot AH, Volovitch M, Henderson CE, Prochiantz A (1993): Antennapedia Homeobox peptide enhances growth and branching of embryonic chicken motoneurons *in vitro*. *J Cell Biol* 120:485-492.
- Bloom FE (1972): The formation of synaptic junctions in developing rat brain. In Pappas GD, Purpura DP (eds): "Structure and Function of Synapses." New York: Raven Press, pp 101-120.
- Boncinelli E, Simeone A, Acampora D, Mavilio F (1991): Hox gene activation by retinoic acid. *Trends Genet* 7:329-334.
- Bovolenta P, Liem RKH, Mason CA (1984): Development of cerebellar astroglia: Transitions in form and cytoskeletal content. *Dev Biol* 102:248-259.
- Bovolenta P, Liem RKH, Mason CA (1987): Glial filament protein expression in astroglia in the mouse visual pathway. *Dev Brain Res* 33:113-126.
- Boyles JK, Pitas RE, Wilson E, Mahley R, Taylor JM (1985): Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J Clin Invest* 76:1501-1513.
- Brittis PA, Canning DR, Silver J (1992): Chondroitin sulfate as a regulator of neuronal patterning in the retina. *Science* 255:733-736.
- Bruckenstein DA, Higgins D (1988): Morphological differentiation of embryonic rat sympathetic neurons in tissue culture. 1. Conditions under which neurons form axons but not dendrites. *Dev Biol* 128:324-336.
- Brugg B, Reddy D, Matus A (1993): Attenuation of microtubule-associated protein 1B expression by antisense oligodeoxynucleotides inhibits initiation of neurite outgrowth. *Neuroscience* 52:489-496.
- Buettner HM, Pittman RN (1991): Quantitative effects of laminin concentration on neurite growth *in vitro*. *Dev Biol* 145:266-276.
- Bullock R, Fujisawa (1992): The role of glutamate antagonists for the treatment of CNS injury. *J Neurotrauma* 9:S443-462.
- Burgess WH, Maciag R (1989): The heparin-binding (fibroblast) growth factor family of proteins. *Ann Rev Biochem* 58:575-606.
- Cáceres A, Banker G, Steward O, Binder L, Payne M (1984a): MAP2 is localized to the dendrites of hippocampal neurons which develop in culture. *Dev Brain Res* 13:314-318.
- Cáceres A, Banker GA, Binder L (1986): Immunocytochemical localization of tubulin and microtubule-associated protein 2 during the development of hippocampal neurons in culture. *J Neurosci* 6:714-722.

- Cáceres A, Binder L I, Payne M R, Bender P, Rebhun L, Steward O (1984b): Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. *J Neurosci* 4:394-410.
- Cáceres A, Mautino J, Kosik K S (1992): Suppression of MAP2 in cultured cerebellar macroneurons inhibits minor neurite formation. *Neuron* 9:607-618.
- Cáceres A, Potrebic S, Kosik K (1991): The effect of tau antisense oligonucleotides on neurite formation of cultured cerebellar macroneurons. *J Neurosci* 11:1515-1523.
- Cameron PL, Sudhof TC, Jahn R, de Camilli P (1991): Colocalization of synaptophysin and transferrin receptors: Implications for synaptic vesicle biogenesis. *J Cell Biol* 115:151-164.
- Cameron RS, Rakic P (1991): Glial cell lineage in the cerebral cortex: a review and synthesis. *Glia* 4:124-137.
- Canady KS, Rubel EW (1992): Rapid and reversible astrocytic reaction to afferent activity blockade in chick cochlear nucleus. *J Neurosci* 12:1001-1009.
- Carbonetto S, Guver MM, Turner DC (1983): Nerve fiber growth in culture on fibronectin, collagen, and glycosaminoglycan substrates. *J Neurosci* 3:2324-2335.
- Carden MJ, Schlaepfer WW, Lee VM-Y (1985): The structure, biochemical properties, and immunogenicity of neurofilament peripheral regions are determined by phosphorylation state. *J Biol Chem* 260:9805-9817.
- Carlstedt T (1985): Dorsal root innervation of spinal cord neurons after dorsal root implantation into the spinal cord of adult rats. *Neurosci Letts* 55:343-348.
- Caroni P, Schwab ME (1988): Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J Cell Biol* 106:1281-1288.
- Cattaneo E, McKay R (1990): Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature* 347:762-765.
- Caveness WF (1979): Incidence of craniocerebral trauma in the United States in 1976 with trend from 1970-1975. *Adv Neurol* 22:1-3.
- Chalazonitis A, Kessler JA, Twardzik DR, Morrison RS (1992): Transforming growth factor α , but not epidermal growth factor, promotes the survival of sensory neurons *in vitro*. *J Neurosci* 12:583-594.
- Chamak B, Fellans A, Glowinski J, Prochiantz A (1987): MAP2 expression and neuritic outgrowth and branching are correlated through region-specific neuro-astroglial interactions. *J Neurosci* 7:3163-3170.
- Chamak B, Prochiantz A (1989): Influence of extracellular matrix proteins in the expression of neuronal polarity. *Development* 106:483-491.
- Chen J, Kanai Y, Cowan NJ, Hirokawa N (1992): Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons. *Nature* 360:674-677.

- Cheng B, Mattson MP (1991): NGF and bFGF protect rat hippocampal and human cortical neurons against hypoglycemic damage by stabilizing calcium homeostasis. *Neuron* 7:1031-1041.
- Chernauek SD (1993): Insulin-like growth factor-I (IGF-I) production by astroglial cells: regulation and importance for epidermal growth factor-induced cell replication. *J Neurosci Res* 34:189-197.
- Chesler M (1990): The regulation and modulation of pH in the nervous system. *Prog Neurobiol* 34:401-427.
- Choi DW, Koh J-Y, Peters S (1988): Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J Neurosci* 8:185-196.
- Choi DW, Maulucci-Gedde M, Kriegstein AR (1987): Glutamate neurotoxicity in cortical cell culture. *J Neurosci* 7:357-368.
- Choi, DW (1988): Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1:623-634.
- Clark B, Mobbs P (1992): Transmitter operated channels in rabbit retinal astrocytes studied in situ by whole cell patch clamping. *J Neurosci* 12:664-673.
- Cleveland DW, Hoffman PN (1991): Neuronal and glial cytoskeletons. *Curr Opin Neurobiol* 1:346-353.
- Cline HT, Constatine-Paton M (1989): The NMDA receptor antagonists disrupt the retinotectal topographic map. *Neuron* 3:413-426.
- Cohen-Corey S, Dreyfus CF, Black IB (1991): NGF and excitatory neurotransmitters regulate survival and morphogenesis of cultured cerebellar Purkinje cells. *J Neurosci* 11:462-471.
- Coleman PD, Flood DG (1986): Dendritic proliferation in the aging brain as a compensatory repair mechanism. *Prog Br Res* 70:227-237.
- Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990): Glutamate induces calcium waves in cultured astrocytes: long range glial signaling. *Science* 247:470-473.
- Corvalan V, Cole R, de Vellis J, Hagiwara S (1990): Neuronal modulation of calcium channel activity in cultured rat astrocytes. *Proc Natl Acad Sci USA* 87:4345-4348.
- Cowan WM, Stanfield BB, Kishi K (1980): The development of the dentate gyrus. *Curr Top Dev Biol* 15:103-157.
- Coyle JT, Bird SJ, Evans RH, Gulley RL, Nadler JV, Nicklas WJ, Olney JW (1981): Excitotoxic amino acid neurotoxins, selectivity, specificity and mechanism of action. *Neurosci Res Prog Bull* 19:331-427.
- Craig AM, Blackstone CD, Hugamir RL, Banker G (1993): The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. *Neuron* 10:1055-1068.
- Craig AM, Jareb M, Banker G (1992): Neuronal polarity. *Curr Opin Neurobiol* 2:602-606.

- Crandall J, Fischer I (1989): Developmental regulation of microtubule-associated protein 2 expression in regions of mouse brain. *J Neurochem* 53:1910-1917.
- Crandall JE, Jacobson M, Kosik KS (1986): Ontogenesis of microtubule-associated protein 2 (MAP2) in embryonic mouse cortex. *Dev Brain Res* 28:127-133.
- Crossin KL, Prieto AL, Hoffman S, Jones FS, Friedlander DR (1990): Expression of adhesion molecules and the establishment of boundaries during embryonic and neural development. *Exp Neurol* 109:6-18.
- Crutcher KA (1989): Tissue sections from mature brain and spinal cord as substrates for neurite growth *in vitro*: extensive growth on gray matter but little on white matter. *Exp Neurol* 104:39-54.
- Dahl D (1981): The vimentin-GFAP protein transition in rat neuroglia cytoskeleton occurs at the time of myelination. *J Neurosci Res* 6:741-748.
- Dahl D, Bignami A (1976): Immunogenic properties of the glial fibrillary acidic protein. *Brain Res* 116:150-157.
- Dahl D, Bignami A (1985): Neurofilament phosphorylation in development. *Exp Cell Res* 162:220-230.
- Dahl D, Crosby C J, Gardener E E, Bignami A (1986): Delayed phosphorylation of the largest neurofilament protein in rat optic nerve development. *J Neurosci Res* 15:513-519.
- Damsky CH, Werb Z (1992): Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* 4:772-781.
- Dani JW, Chernjavsky A, Smith SJ (1992): Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* 8:429-440.
- David S, Bouchard C, Tsatas O, Giftochristos N (1990): Macrophages can modify the nonpermissive nature of the adult mammalian central nervous system. *Neuron* 5:463-469.
- Davis L, Banker GA, Steward O (1987): Selective dendritic transport of RNA in hippocampal neurons in culture. *Nature* 330:477-479.
- De Koninck P, Carbonetto S, Cooper E (1993): NGF induces neonatal rat sensory neurons to extend dendrites in culture after removal of satellite cells *J Neurosci* 13:577-585.
- De Leeuw AM, Gaur VP, Saari JC, Milam AH (1990): Immunolocalization of cellular retinol-, retinaldehyde and retinoic binding proteins in the retina during pre- and postnatal development. *J Neurocytol* 19:253-264.
- DeCamilli P, Miller P, Navove F, Theurkau W E, Vallee R B (1984): Distribution on microtubule-associated protein 2 (MAP2) in the nervous system of the rat studied by immunofluorescence. *Neuroscience* 11:819-946.
- Deitch JA, Rubel EW (1984): Afferent influences on brain stem auditory nuclei of the chicken: Time course and specificity of dendritic atrophy following deafferentation. *J Comp Neurol* 229:66-79.

- Deitch JS, Banker GA (1993): An electron microscopic analysis of hippocampal neurons developing in culture: early stages in the emergence of polarity. *J Neurosci* 13:4301-4315.
- Denis-Donini S, Estenoz M (1988): Interneurons versus efferent neurons: heterogeneity in their neurite outgrowth response to glia from several brain regions. *Dev Biol* 130:237-249.
- Denis-Donini S, Glowinski J, Prochiantz A (1984): Glial heterogeneity may define the three-dimensional shape of mouse mesencephalic dopaminergic -neurons. *Nature* 307:641-643.
- Deschepper CF, Biogornia V, Berens ME, Lapointe MC (1991): Production of thrombin and antithrombin III by brain and astroglial cell cultures. *Mol Brain Res* 11:355-358.
- DeWitt DA, Silver J, Canning DR, Perry G (1993): Chondroitin sulfate proteoglycans are associated with the lesions of Alzheimer's disease. *Exp Neurol* 121:149-152.
- Díaz-Nido J, Montoro RJ, López-Barneo J, Avila J (1993): High external potassium induces an increase in the phosphorylation of the cytoskeletal protein MAP2 in rat hippocampal slices. *Eur J Neurosci* 5:818-824.
- Diedrich JF, Minnigan H, Carp RI, Whittaker JN, Race R, Frey W, Haase AT (1991): Neuropathological changes in scrapie and Alzheimer's disease associated with an increased expression of apolipoprotein E and cathepsin in astrocytes. *J Virol* 65:4759-4768.
- Dinsmore J, Solomon F (1991): Inhibition of MAP2 expression affects both morphological and cell division phenotypes of neuronal differentiation. *Cell* 64:817-826.
- Djabali K, Zissopoulou A, de Hoop MJ, Georgatos SD, Dotti CG (1993): Peripherin expression in hippocampal neurons induced by muscle soluble factor(s). *J Cell Biol* 123:1197-1206.
- Dodd J, Jessell TM (1988): Axon guidance and the patterning of neuronal projections in vertebrates. *Science* 242:692-699.
- Doering LC (1992): Appropriate target interactions prevent abnormal cytoskeletal changes in neurons: a study with intra-sciatic grafts of the septum and the hippocampus. *J Neurosci* 12:3399-3413.
- Doherty P, Cohen J, Walsh FS (1990): Neurite outgrowth in response to transfected N-CAM changes during development and is modulated by polysialic acid. *Neuron* 5:209-219.
- Doherty P, Rimon G, Mann DA, Walsh FS (1992): Alternative splicing of the cytoplasmic domain of neural cell adhesion molecule alters its ability to act as a substrate for neurite outgrowth. *J Neurochem* 58:2338-2341.
- Doherty P, Walsh FS (1992): Cell adhesion molecules, second messengers and axonal growth. *Curr Opin Neurobiol* 2:595-601.
- Dotti C G, Banker GA, Binder LI (1987): The expression of and distribution of microtubule-associated protein 2 in hippocampal neurons in the rat in situ and in cell culture. *Neuroscience* 23:121-130.
- Dotti C, Banker G (1992): Intracellular organization of hippocampal neurons during development of neuronal polarity. *J Cell Sci* 15:75-84.

- Dotti C, Simons K (1990): Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell* 62:63-72.
- Dotti CG, Parton RG, Simons K (1991): Polarized sorting of glypiated proteins in hippocampal neurons. *Nature* 349:158-161.
- Dotti CG, Sullivan CA, Banker GA (1988): The establishment of polarity by hippocampal neurons in culture. *J Neurosci* 8:1454-1458.
- Doucette R (1990): Glial influences on axonal growth in the primary olfactory system. *Glia* 3:433-449.
- Dupouey P, Benjelloun-Touini D, Gomes D (1985): Histochemical demonstration of an organized cytoarchitecture of the radial glia in the CNS of the embryonic mouse. *Dev Neurosci* 7:81-93.
- Dutly F, Schwab ME (1991): Neurons and astrocytes influence the development of purified O-2A progenitor cells. *Glia* 4:559-571.
- Dyken M, Wolf PA, Barnett HJM, Began JJ, Hass WK, Kurtzke JF, Sundt TM (1984): Risk factors in stroke. *Stroke* 15:1105-1111.
- Eddleston M, Mucke L (1993): Molecular profile of reactive astrocytes--implication for their role in neurologic disease. *Neuroscience* 54:15-36.
- Edelman GM (1987): CAMs and Igs: Cell adhesion and the evolutionary origins of immunity. *Immunol Rev* 100:11-45.
- Edwards MA, Yamamoto M, Caviness VS Jr (1990): Organization of radial glia and related cells in the developing murine CNS: an analysis based upon a new monoclonal antibody marker. *Neuroscience* 36:121-144.
- Emoto M, Gonzalez AM, Walicke PA, Wada E, Simmons DM, Shimasaki S, Baird A (1989): Basic fibroblast growth factor (FGF) in the central nervous system: identification of specific loci of basic FGF expression in the rat brain. *Growth Factors* 2:21-29.
- Eng LF (1985): Glial fibrillary acidic protein: the major protein of glial intermediate filaments in differentiated astrocytes. *J Neuroimmunol* 8:203-214.
- Engle J, Bohn MC (1991): The neurotrophic effects of fibroblast growth factors on dopaminergic neurons *in vitro* are mediated by mesencephalic glia. *J Neurosci* 11:3070-3078.
- Evans DA, Funkenstein HH, Albert MS, Scherr PA, Cook NR, Chown MJ, Hebert LE, Hennekens CH, Taylor JO (1989): Prevalence of Alzheimer's disease in a community population of older persons. Higher than previously reported. *JAMA* 262:2551-2556.
- Fallon JH, Annis CM, Gentry LE, Twardzik DR, Loughlin SE (1990): Localization of cells containing transforming growth factor- α precursor immunoreactivity in the basal ganglia of the adult rat brain. *Growth Factors* 2:241-250.
- Fallon JH, Seroogy KB, Loughlin SE, Morrison RS, Bradshaw RA, Knauer DJ, Cunningham DD (1984): Epidermal growth factor immunoreactive material in the central nervous system: location and development. *Science* 224:1107-1109.

- Fallon JR (1985): Preferential outgrowth of central nervous system neurites on astrocytes and Schwann cells as compared with non-glial cells *in vitro*. J Cell Biol 100:198-207.
- Feldman ML, Dowd C (1975): Loss of dendritic spines in aging cerebral cortex. Anat Embryol 148:279-301.
- Ferrara N, Ousley F, Gospodarowicz D (1988): Bovine brain astrocytes express basic fibroblast growth factor, a neurotrophic angiogenic mitogen. Brain Res 462:223-232.
- Ferreira A, Caceres A, Kosik KS (1993): Intraneuronal compartments of the amyloid precursor protein. J Neurosci 13:3112-3123.
- French-Constant C, Miller RH, Kruse J, Schachner M, Raff MC (1986): Molecular specialization of astrocyte processes at nodes of Ranvier in rat optic nerve. J Cell Biol 102:844-852.
- Finkbeiner SM (1993): Glial calcium. Glia 9:83-104.
- Finklestein SP, Apostolides PJ, Caday CG, Prosser J, Philips MF, Klagsbrun M (1988): Increased basic fibroblast growth factor (bFGF) immunoreactivity at the site of focal brain wounds. Brain Res 460:253-259.
- Fischer I, Shea JB, Sapirstein VS, Kosik KS (1986): Expression and distribution of microtubule associated protein 2 in neuroblastoma and primary neuronal cells. Dev Brain Res 25:99-109.
- Fishman PS, Nilaver G, Kelly JP (1983): Astrogliosis limits the integration of peripheral nerve grafts into the spinal cord. Brain Res 277:175-180.
- Flanagan JG, Chan DC, Leder P (1991): Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the *Sl^d* mutant. Cell 64:1025-1035.
- Fletcher TL, Cameron P, De Camilli P, Banker G (1991): The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. J Neurosci. 11:1617-1626.
- Flott B, Seifert W (1991): Characterization of glutamate uptake systems in astrocyte primary cultures from rat brain. Glia 4:293-304.
- Fok-Seang J, Miller RH (1992): Astrocyte precursors in neonatal rat spinal cord cultures. J Neurosci 12:2751-2764.
- Foster GA, Dahl D, Lee VM-Y (1987): Temporal and topographic relationships between the phosphorylated and nonphosphorylated epitopes of the 200 kDa neurofilament protein during development *in vitro*. J Neurosci 7:2651-2663.
- Frederiksen K, McKay RDG (1988): Proliferation and differentiation of rat neuroepithelial precursor cells *in vivo*. J Neurosci 8:1144-1151.
- Frei T, von Bohlen und Halbach F, Wille W, Schachner M (1992): Different extracellular domains of the neural cell adhesion molecule (NCAM) are involved in different functions. J Cell Biol 118:177-194.

- Frim DM, Short MP, Rosenberg WS, Simpson J, Breakefield XO, Isacson O (1993): Local protective effects of nerve growth factor-secreting fibroblasts against excitotoxic lesions in the rat striatum. *J Neurosurg* 78:267-273.
- Furley AJ, Morton SB, Manalo D, Karagogeos D, Dodd J, Jessell T (1990): The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite promoting activity. *Cell* 61:157-170.
- Furukawa S, Furukawa Y, Satoyoshi E, Hayashi K (1986): Synthesis and secretion of nerve growth factor by mouse astroglial cells in culture. *Biochem Biophys Res Commun* 136:57-63.
- Gage FH, Olejniczak P, Armstrong DM (1988): Astrocytes are important for sprouting in the septohippocampal circuit. *Exp Neurol* 102:2-13.
- Gallo V, Patrizio M, Levi G (1991): GABA release triggered by the activation of neuron like non-NMDA receptors in cultured type 2 astrocytes is carrier mediated. *Glia* 4:245-255.
- Garcia-Estrada J, Garcia-Segura LM, Torres-Aleman I (1992): Expression of insulin-like growth factor I by astrocytes in response to injury. *Brain Res* 592:343-347.
- Gasser UE, Hatten ME (1990): Neuron-glia interactions of rat hippocampal cells *in vitro*. Glial-guided neuronal migration and neuronal regulation of glial differentiation. *J Neurosci* 10:1276-1285.
- Giulian D (1993): Reactive glia as rivals in regulating neuronal survival. *Glia* 7:102-110.
- Giulian D, Baker TJ (1985): Peptides released by microglia regulate astrocyte proliferation. *J Cell Biol* 101:2411-2415.
- Giulian D, Baker TJ (1986): Characterization of ameboid microglia isolated from developing mammalian brain. *J Neurosci* 6:2163-2178.
- Giulian D, Chen J, Ingeman JE, George JK, Noponen M (1989): The role of mononuclear phagocytes in wound healing after traumatic injury to adult mammalian brain. *J Neurosci* 9:4416-4429.
- Giulian D, Lachman LB (1985): Interleukin-1 stimulation of astroglial proliferation after brain injury. *Science* 228:497-499.
- Giulian D, Robertson C (1990): Inhibition of mononuclear phagocytes reduces ischemic injury in the spinal cord. *Ann Neurol* 27:33-42.
- Giulian D, Vaca K, Corpuz M (1993): Brain glia release factors with opposing actions upon neuronal survival. *J Neurosci* 13:29-37.
- Giulian D, Young DG, Woodward J, Brown DC, Lachman LB (1988): Interleukin-1 is an astroglial growth factor in the developing brain. *J. Neurosci* 8:709-714.
- Globus A, Rosenzweig MR, Bennett EL, Diamond MC (1973): Effects of differential experience on dendritic spine counts in the rat cerebral cortex. *J Comp Physiol Psychol* 82:175-181.
- Gluckman P, Klempf N, Guan J, Mallard C, Sirimanne E, Dragunow MK, Singh K, Williams C, Nikolics K (1992): A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun* 182:593-599.

- Goldman JE, Vaysse PJ-J (1991): Tracing glial lineages in the mammalian forebrain. *Glia* 4:149-156.
- Goldstein LA, Kur EM, Sengelaub DR (1990): Androgen regulation of dendritic growth and retraction in the development of a sexually dimorphic nucleus. *J Neurosci* 10:935-946.
- Golgi C (1873): Sulla struttura della sostanza grigia del cervello. *Gazz Med Ital Lombardo* 6:41-56.
- Gómez-Pinilla F, Knauer DJ, Nieto-Sampedro M (1988): Epidermal growth factor receptor immunoreactivity in rat brain. Development and cellular localization. *Brain Res* 438:385-390.
- Gómez-Pinilli F, Lee JW-K, Cotman CW (1992): Basic FGF in adult rat brain: cellular distribution and response to enterorhinal lesion and fimbria-fornix transection. *J Neurosci* 12:345-355.
- Gonzalez A-M, Buscaglia M, Ong M, Baird A (1990): Distribution of basic fibroblast growth factor in the 18-day rat fetus: localization in the basement membranes of diverse tissues. *J Cell Biol* 110:753-765.
- Goodman CS, Shatz CJ (1993): Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell* 72:77-98.
- Goslin K, Banker G (1990): Rapid changes in the distribution of gap-43 correlate with the expression of neuronal polarity during normal development and under experimental conditions. *J Cell Biol* 110:1319-1331.
- Goslin K, Schreyer DJ, Skene JHP, Banker G (1990): Changes in the distribution of GAP-43 during the development of neuronal polarity. *J Neurosci* 10:588-602.
- Gospodarowicz D (1991): Biological activities of fibroblast growth factors. *Ann NY Acad Sci* 638:1-8.
- Gospodarowicz D, Neufeld G, Schweigerer L (1986): Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. *Cell Differentiation* 19:1-17.
- Grady MS, Jane JA, Steward O (1989): Synaptic reorganization within the human central nervous system following injury. *J Neurosurg* 71:534-537.
- Greenough WT, Juraska JM, Volkmar FR (1979): Maze training effects on dendritic branching in occipital cortex of adult rats. *Behav Neurol Biol* 26:287-297.
- Greenough WT, Larson JR, Withers GS (1985): Effects of unilateral and bilateral training in a reaching task on dendritic branching of neurons in the rat motor sensory forelimb cortex. *Behav Neural Biol* 44:301-314.
- Grove EA, Kirkwood TBL, Price J (1992): Neuronal precursor cells in the rat hippocampal formation contribute to more than one cytoarchitectonic area. *Neuron* 8:217-229.
- Groves AK, Entwistle A, Jat PS, Noble M (1993): The characterization of astrocyte cell lines that display properties of glial scar tissue. *Dev Biol* 159:87-104.

- Halpain S, Greengard P (1990): Activation of NMDA receptors induces rapid dephosphorylation of the cytoskeletal protein MAP2. *Neuron* 5:237-246.
- Hammer RP, Lindsay RD, Scheibel AB (1981): Development of the brain stem reticular core: an assessment of dendritic state and configuration in the perinatal rat. *Dev Brain Res* 1:179-190.
- Hanks SD, Flood DG (1991): Region-specific stability of dendritic extent in normal human aging and regression in Alzheimer's disease. I. CA1 of hippocampus. *Brain Res* 540:63-82.
- Hansson E (1988): Astroglia from defined brain regions as studied with primary cultures. *Prog Neurobiol* 30:369-397.
- Harbaugh RE (1989): Novel CNS-directed drug delivery systems in Alzheimer's disease and other neurological disorders. *Neurobiol Aging* 10:623-629.
- Harris KM, Stevens JK (1988): Study of dendritic spines by serial electron microscopy and three-dimensional reconstructions. In Lasek RJ, Black MM (eds): "Intrinsic Determinants of Neuronal Form and Function." New York: Alan R. Liss, pp 179-199.
- Harrison RG (1910): The outgrowth of the nerve fiber as a mode of protoplasmic movement. *J Exp Zool* 9:787-848.
- Hartley DM, Kurth MC, Bjerkness L, Weiss JH, Choi DW (1993): Glutamate receptor-induced $^{45}\text{Ca}^{2+}$ accumulation in cortical cell culture correlates with subsequent neuronal degeneration. *J Neurosci* 13:1993-2000.
- Hatten ME (1984): Embryonic cerebellar astroglia *in vitro*. *Brain Res* 315:309-312.
- Hatten ME (1985): Neuronal regulation of astroglial morphology and proliferation *in vitro*. *J Cell Biol* 100:384-396.
- Hatten ME, Liem RKH, Shelandki ML, Mason CA (1991): Astroglia in CNS injury. *Glia* 4:233-243.
- Hatten ME, Lynch M, Ryder RE, Sanchez, Joseph-Silverstein J, Moscattelli D, Rifkin DB (1988): *In vitro* neurite extension by granule neurons is dependent upon astroglial-derived fibroblast growth factor. *Dev Biol* 125:280-289.
- Hauser KF, McLaughlin PJ, Zagon IS (1987): Endogenous opioids regulate dendritic growth and spine formation in developing rat brain. *Brain Res* 416:157-161.
- Hayes, RL, Jenkins LW, Lyeth BG (1992): Neurotransmitter-mediated aspects of traumatic brain injury, acetyl choline and excitatory amino acids. *J Neurotrauma* 9:S173-187.
- Hefti F, Hartikka J, Knusel B (1989): Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. *Neurobiol Aging* 10:515-533.
- Heiter E, Ayala J, Deneffe P, Bousseau A, Rouget P, Mallat M, Prochiantz A (1988): Brain macrophages synthesize interleukin-1 and interleukin-1 mRNA *in vitro*. *J Neurosci Res* 21:391-397.

- Heitler E, Ayala J, Bousseau A, Deneefle P, Prochiantz A (1990): Amoeboid microglial cells and not astrocytes synthesize TNF- α in Swiss mouse brain cell cultures. *Eur J. Neurosci* 2:762-768.
- Herndon ME, Lander AD (1990): A diverse set of developmentally regulated proteoglycans is expressed in the rat central nervous system. *Neuron* 4:949-961.
- Hertz L, McFarlin DE, Waksman BH (1990): Astrocytes: auxiliary cells for immune responses in the central nervous system? *Immunol Today* 11:265-268.
- Higgins D, Waxman A, Banker G (1988): The distribution of microtubule-associated protein 2 changes when dendritic growth is induced in rat sympathetic neurons *in vitro*. *Neuroscience* 24:583-592.
- Hirano M, Goldman JE (1988): Gliogenesis in rat spinal cord: evidence for origin of astrocytes and oligodendrocytes from radial precursors. *J Neurosci Res* 21:155-16.
- Hoffman PN, Cleveland DW, Griffin JW, Landes PW, Cowan NJ, Price DL (1987): Neurofilament gene expression: a major determinant of axonal caliber. *Proc Natl Acad Sci USA* 84:472-3476.
- Hoffman PN, Lasek RJ (1975): The slow component of axonal transport: Identification of the major structural polypeptides of the axon and their generality among mammalian neurons. *J Cell Biol* 66:361-366.
- Hoffman PN, Thompson GW, Griffin JW, Price DL (1985): Changes in neurofilament transport coincide temporally with alterations in the caliber of axons in regenerating motor fibers. *J Cell Biol* 101:1332-1340.
- Holder N, Clarke JDW (1988): Is there a correlation between continuous neurogenesis and directed axon regeneration in the vertebrate nervous system? *TINS* 11:94-99.
- Holzwarth JA, Gibbons SJ, Brorson JR, Philipson LH, Miller RJ (1994): Glutamate receptor agonists stimulate diverse calcium responses in different types of cultured rat cortical glial cells. *J Neurosci* 14:1879-1891.
- Huber G, Matus A (1984): Differences in the cellular distribution of two microtubule-associated proteins, MAP1 and MAP2, in rat brain. *J Neurosci* 4:151-160.
- Huff KR, Schreier W (1990): Fibroblast growth factor inhibits epidermal growth factor-induced responses in rat astrocytes. *Glia* 3:193-204.
- Hunter DD, Cashman N, Morris-Valero R, Bullock JW, Adams SP, Sanes JR (1991): An LRE (leucine-arginine-glutamate)-dependent mechanism for adhesion of neurons to S-laminin. *J Neurosci* 11:3960-3971.
- Hynes RO (1992): Integrins: versatility, modulation and signaling in cell adhesion. *Cell* 69:11-25.
- Ihara Y (1988): Massive somatodendritic sprouting of cortical neurons in Alzheimer's disease. *Brain Res* 459:138-144.
- Inetebi AD, Flaxman MS, Ganong WF, Deschepper CF (1990): Angiotensinogen production by rat astroglial cells *in vitro* and *in vivo*. *Neuroscience* 34:545-554.

- Ingraham CA, McCarthy KD (1989): Plasticity of process-bearing glial cell cultures from neonatal rat cerebral cortical tissue. *J Neurosci* 9:63-69.
- Isacson O, Sofroniew MV (1992): Neuronal loss or replacement in the injured adult cerebral neocortex induces extensive remodeling of intrinsic and afferent neural systems. *Exp Neurol* 117:151-175.
- Jacobson M (1991): "Developmental Neurobiology (3rd ed)." New York: Plenum Press.
- Janzer RC, Raff MC (1987): Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* 325:253-257.
- Johnson GV, Litsky JM, Jope RS (1991): Degradation of microtubule associated protein-2 and brain spectrin by calpain: A comparative study. *J Neurochem* 56:1630-1638.
- Johnson MI, Higgins D, Ard MD (1989): Astrocytes induce dendritic development in cultured sympathetic neurons. *Dev Brain Res* 47:289-292.
- Johnson-Green PC, Dow KE, Riopelle RJ (1992): Neurite growth modulation associated with astrocyte proteoglycans: influence of activators of inflammation. *Glia* 5:33-42.
- Joliot AH, Pernelle C, Deagostini-Bazin H, Prochiantz A (1991): Antennapedia Homeobox peptide regulates neural morphogenesis. *Proc Natl Acad Sci USA* 88:1864-1868.
- Jones TA, Schallert T (1994): Use-dependent growth of pyramidal neurons after neocortical damage. *J Neurosci* 14:2140-2152.
- Joo F (1987): Current aspects of the development of the blood brain barrier. *Int J Dev Neurosci* 5:369-372.
- Kalderon N, Ahonen K, Federoff S (1990): Developmental transition in plasticity properties of differentiating astrocytes: Age related biochemical profile of plasminogen activators in astroglial cultures. *Glia* 3:413-426.
- Kalsbeek WD, McLaurin RL, Harris BJ, Miller JD (1980): The National Head and Spinal Cord Injury Survey: Major findings. *J. Neurosurg* 53: S19-S31.
- Kandel ER, Schwarz JH, Jessel TM (1991): "Principles of Neural Science (3rd ed)." New York: Elsevier.
- Kaneko T, Shigemoto R, Mizuno N (1988): Metabolism of glutamate and ammonia in astrocyte: an immunocytochemical study. *Brain Res* 457:160-164.
- Kapfhammer JP, Schwab ME (1992): Modulators of neuronal migration and neurite growth. *Curr Opin Cell Biol* 4:863-868.
- Katayama Y, Becker DP, Tamura T (1990): Massive increases in extracellular potassium and the indiscriminate release of glutamate following concussive brain injury. *J Neurosurg* 73:889-900.
- Kawaja MD, Gage FH (1991): Reactive astrocytes are substrates for the growth of adult CNS axons in the presence of elevated levels of nerve growth factor. *Neuron* 7:1019-1030.

- Killisch I, Dotti C, Laurie DJ, Luddens DJ, Seeburg PH (1991): Expression patterns of GABA receptor subtypes in developing hippocampal neurons. *Neuron* 7:927-936.
- Kimelberg HK (1983): Primary astrocyte cultures - a key to astrocyte function. *Cell Mol Neurobiol* 3:1-16.
- Kimelberg HK (1992): Astrocytic edema in CNS trauma. *J Neurotrauma* 9(suppl):S71-S81.
- Kinoshita Y, Kinoshita C, Heuer JG, Bothwell M (1993): Basic fibroblast growth factor promotes adhesive interactions of neuroepithelial cells from chick neural tube with extracellular matrix proteins in culture. *Development* 119:943-956.
- Kirschner M, Mitchison T (1986): Beyond self-assembly: from microtubules to morphogenesis. *Cell* 45:329-342.
- Kitagawa K, Matsumoto M, Niinobe M, Mikishibe K, Hata R, Ueda H, Handa N, Fukunaga R, Isaka Y, Kimura K, Kamada R (1989): Microtubule-associated protein 2 as a sensitive marker for cerebral ischemic damage - immunohistochemical investigation of dendritic damage. *Neuroscience* 31:401-411.
- Kitamura T, Tsuchihashi Y, Fujita S (1978): Initial response of silver-impregnated resting microglia to stab wounding in the rabbit hippocampus. *Acta Neuropathol (Berl)* 44:31-39.
- Kleinman R, Banker G, Steward O (1990): Differential subcellular localization of particular mRNAs in hippocampal neurons in culture. *Neuron* 5:821-830.
- Kleinman R, Banker G, Steward O (1994): Development of subcellular mRNA compartmentation in hippocampal neurons in culture. *J Neurosci* 14:1130-1140.
- Kliot M, Smith GM, Siegal JD, Silver J (1990): Astrocyte-polymer implants promote regeneration of dorsal root fibers into the adult mammalian spinal cord. *Exp Neurol* 109:57-69.
- Knops J, Kosik K, Lee G, Pardee J, Cohen-Gould L, McConlogue L (1991): Overexpression of tau in a non-neuronal cell induces long cellular processes. *J Cell Biol* 114:725-533.
- Koch C, Zador A (1993): The function of dendritic spines: devices subserving biochemical rather than electrical compartmentalization. *J Neurosci* 13:413-422.
- Kolb B, Gibb R (1991): Environmental enrichment and cortical injury: behavioral and anatomical consequences of frontal cortex lesions. *Cereb Cortex* 1:189-198.
- Kosik KS, Finch EA (1987): MAP2 and tau segregate into dendritic and axonal domains after the elaboration of morphologically distinct neurites: an immunocytochemical study of cultured rat cerebrum. *J Neuroscience* 7:3142-3153.
- Kromer LF (1986): Nerve growth factor treatment after brain injury prevents neuronal death. *Science* 235:214-216.
- Kruse J, Keilhauer G, Faissner A, Timpl R, Schachner M (1985): The J1 glycoprotein - a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature* 316:145-148.
- Kuffler SW, Nicholls JG, Martin AR (1984): From neuron to brain: A cellular approach to the function of the Nervous system. Sunderland, Sinauer.

- Kuffler SW, Nicholls JG, Orkand RK (1966): Physiologic properties of glial cells in the central nervous system of amphibia. *J Neurophysiol* 29:768-787.
- Kurtzke JF (1975): Epidemiology of spinal cord injury. *Exp Neurol* 48:163-236.
- Kurtzke JF (1983): Epidemiology and risk factors in thrombotic brain infarct. In: Harrison MJG, Dyken MC (eds). *Cerebrovascular Disease*. Butterworth, London, pp 27-45.
- LaFont F, Rouget M, Rousselet A, Valenza C, Prochiantz A (1993): Specific responses of axons and dendrites to cytoskeleton perturbations: an *in vitro* study. *J Cell Sci* 104:433-443.
- LaFont F, Rouget M, Triller A, Prochiantz A, Rousselet A (1992): *In vitro* control of neuronal polarity by glycosaminoglycans. *Development* 114:17-29.
- Lagenaur C, Kunemund V, Fischer G, Fushiki S, Schachner M (1992): Monoclonal M6 antibody interferes with neurite extension of cultured neurons. *J Neurobiol* 23:71-88.
- Landis DMD, Weinstein LA, Skordeles CJ (1990): Serum influences the differentiation of membrane structure in cultured astrocytes. *Glia* 3:212-221.
- Landry CF, Ivy GO, Brown IR (1990): Developmental expression of glial fibrillary acidic protein mRNA in the rat brain analyzed by *in situ* hybridization. *J Neurosci Res* 25:194-203.
- Lasek RJ (1988): Studying the intrinsic determinants of neuronal form and function. In Lasek RJ, Black MM (eds): "Intrinsic Determinants of Neuronal Form and Function." New York: Alan R. Liss, pp 3-58.
- Lauder JM (1983): Hormonal and humoral influences on brain development. *Psychoneuroendocrinology* 8:121-155.
- Laywell ED, Dorries U, Bartsch U, Faissner A, Schachner M, Steindler DA (1992): Enhanced expression of the developmentally regulated extracellular matrix molecule tenascin following adult brain injury. *Proc Natl Acad Sci USA* 89:2634-2638.
- Lazar LM, Blum M (1992): Regional distribution and developmental expression of epidermal growth factor and transforming growth factor- α mRNA in mouse brain by a quantitative nuclease protection assay. *J Neurosci* 12:1688-1697.
- Le Roux I, Joliot AH, Bloch-Gallego E, Prochiantz A, Volovitch M (1993): Neurotrophic activity of the Antennapedia Homeodomain depends on its specific DNA-binding properties. *Proc Natl Acad Sci USA* 90:9120-9124.
- Lee G (1993): Non-motor microtubule-associated proteins. *Curr Opin Cell Biol* 5:88-94.
- Lein PJ, Higgins D (1989): Laminin and basement membrane extract have different effects on axonal and dendritic outgrowth from embryonic rat sympathetic neurons *in vitro*. *Dev Biol* 136:330-345.
- Lemmon V, Burden SM, Payne HR, Gwendolynne JE, Hlavin ML (1992): Neurite growth on different substrates: permissive versus instructive influences and the role of adhesive strength. *J Neurosci* 12:818-826.

- Lerea LS, McCarthy KD (1989): Astroglial cells *in vitro* are heterogeneous with respect to expression of the $\alpha 1$ -adrenergic receptor. *Glia* 2:135-147.
- Letourneau PC, Condic ML, Snow DM (1994): Interactions of developing neurons with the extracellular matrix. *J Neurosci* 14:915-928.
- Leventhal AG, Ault SJ, Vitek DJ, Shou T (1989): Extrinsic determinants of retinal ganglion cell development in primates. *J Comp Neurol* 286:170-189.
- Levi-Montalcini T (1987): The nerve growth factor 35 years later. *Science* 237:1154-1161.
- Levine RB, Truman JW (1985): Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. *J Neurosci* 5:2424-2431.
- Levison SW, McCarthy KD (1991): Astroglia in culture. In: "Culturing Nerve Cells." Cambridge, MA: MIT Press, pp 309-336.
- Levitt P, Rakic P (1980): Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. *J Comp Neurol* 193:815-840.
- Lewis DA, Higgins GA, Young WG, Goldgaber D, Gajdusek DC, Wilson MC, Morrison JH (1989): Distribution of precursor amyloid protein messenger RNA in the human cerebral cortex: relationship to neuritic plaques and neurofibrillary tangles. *Proc Natl Acad Sci USA* 85:1691-1695.
- Liesi P, Dahl D, Vaheri A (1983): Laminin is produced by early rat astrocytes in primary culture. *J Cell Biol* 96:920-24.
- Liesi P, Kirkwood T, Vaheri A (1986): Fibronectin is expressed by astrocytes cultured from embryonic and early postnatal rat brain. *Exp Cell Res* 63:175-85.
- Lieth E, McClay DR, Lauer JM (1990): Neuronal-glial interactions: complexity of neurite outgrowth correlates with substrate adhesivity of serotonergic neurons. *Glia* 3:169-179.
- Lillien LE, Raff MC (1990): Differentiation signals in the CNS: type-2 astrocyte development *in vitro* as a model system. *Neuron* 5:111-119.
- Lillien LE, Sendtner M, Rohrer H, Hughes SM, Raff MC (1988): Type-2 astrocyte development in rat brain cultures initiated by a CNTF-like protein produced by type-1 astrocytes. *Neuron* 1:485-494.
- Lim R, Miller JF, Hicklin DJ, Andresen AA (1985): Purification of bovine glia maturation factor and characterization with monoclonal antibody *Biochemistry* 24:8070-8074.
- Lindholm D, Castren E, Tsoulfas P, Kolbeck R, Berzaghi MP, Leingartner A, Heisenberg CP, Tessarollo L, Parada LF, Thoenen H (1993): Neurotrophin-3 induced by tri-iodothyronine in cerebellar granule cells promotes Purkinje cell differentiation. *J Cell Biol* 122:443-450.
- Lindvall O (1991): Prospects of transplantation in human neurodegenerative diseases. *TINS* 14:376-384.
- Lipton SA (1989): Growth factors for neuronal survival and process regeneration. Implications in the mammalian central nervous system. *Arch Neurol* 46:1241-1248.

- Lipton SA, Kater SB (1989): Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *TINS* 12:265-270.
- Lipton SA, Rosenberg PA (1994): Excitatory amino acids as a final common pathway for neurologic disorders. *N Eng J Med* 330:613-622.
- Liuzzi FJ, Lasek RJ (1987): Astrocytes block axonal regeneration in mammals by activating the physiologic stop pathway. *Science* 237:642-645.
- Lochter A, Schachner (1993): Tenascin and extracellular matrix glycoproteins: from promotion to polarization of neurite outgrowth *in vitro*. *J Neuroscience* 13:3986-4000.
- Lochter A, Vaughan L, Kaplony A, Prochiantz A, Schachner M, Faissner A (1991): J1/tenascin in substrate bound and soluble form displays contrary effects on neurite outgrowth. *J Cell Biol* 113: 1159-1171.
- Logan A, Grautschy SA, Gonzalez A-M, Baird A (1992): A time course for the focal elevation of synthesis of basic fibroblast growth factor and one of its high-affinity receptors (flg) following a localized cortical brain injury. *J Neurosci* 12:3828-3837.
- Lotz MM, Bursdal CA, Erickson HP, McClay DR (1989): Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J Cell Biol* 109:1795-1805.
- Lowry OH, Passonneau JV, Hasselberger FX, Schulz DW (1964): Effect of ischemia on known substrates and cofactors of glycolytic pathway in brain. *J Biol Chem* 239:18-30.
- Lu B, Yokoyama M, Dreyfus CF, Black IB (1991): NGF gene expression in actively growing brain glia. *J Neurosci* 11:318-326.
- Lugaro E (1907): Sulle funzioni della neuroglia. *Riv Patol Nev Ment* 12:225-233.
- Lund RD, Chang FLF, Hankin MH, Lagenaur CF (1985): Use of a species-specific antibody for demonstrating mouse neurons transplanted to rat brains. *Neurosci Lett*, 61:221-226.
- Lynch G (1986): "Synapses, Circuits, and the Beginnings of Memory." Cambridge, MA: MIT Press.
- Madrazo I, Franco-Bourland R, Aguilera M, Ostrosky-Solis F, Cuevas C, Castrejon H, Magallon E, Madrazo M (1991): Development of human neural transplantation. *Neurosurgery* 29:165-177.
- Maiese K, Boniece I, DeMeo D, Wagner JA (1993): Peptide growth factors protect against ischemia in culture by preventing nitric oxide toxicity. *J Neurosci* 13:3034-3040.
- Manthorpe M, Nieto-Sampedro M, Skaper SD, Lewis ER, Barbin G, Longo FM, Cotman CW, Varon S (1983): Neuronotrophic activity in brain wounds of the developing rat. Correlation with implant survival in the wound cavity. *Brain Res* 267:47-56.
- Mapstone TB (1991): Expression of platelet-derived growth factor and transforming growth factor and their correlation with cellular morphology in glial tumors. *J Neurosurg* 75:447-451.
- Martin DL (1992): Synthesis and release of neuroactive substances by glial cells. *Glia* 5:81-94.

- Marvin M, McKay R (1992): Multipotential stem cells in the vertebrate CNS. *Cell Biol* 3:401-411.
- Mason CA, Edmonson JC, Hatten ME (1988): The extending astroglial process: development of glial cell shape, the growing tip, and interactions with neurons. *J Neurosci* 8:3124-3134.
- Mathewson AJ, Berry M (1985): Observations on the astrocyte response to a cerebral stab wound in adult rats. *Brain Res* 327:61-69.
- Mattheissen HP, Schamlenbach C, Müller HW (1989): Astroglia-released neurite growth-inducing activity for embryonic hippocampal neurons is associated with laminin bound in a sulfated complex and free fibronectin. *Glia* 2:177-188.
- Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE (1992): b-amyloid peptide destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J Neurosci* 12:376-389.
- Mattson MP, Dou P, Kater SB (1988a): Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J Neurosci* :2087-2100.
- Mattson MP, Kumar KN, Wang H, Cheng B, Michaelis EK (1993): Basic FGF regulates the expression of a functional 71kDa NMDA receptor protein that mediates calcium influx and neurotoxicity in hippocampal neurons. *J Neurosci* 13:4575-4588.
- Mattson MP, Lee RE, Adams ME, Guthrie PB, Kater SB (1988b): Interaction between entorhinal axons and target hippocampal neurons: a role for glutamate in the development of hippocampal circuitry. *Neuron* 1:865-876.
- Mattson MP, Murrain M, Guthrie PB, Kater SB (1989): Fibroblast growth factor and glutamate: opposing roles in the generation and degeneration of hippocampal neuroarchitecture. *J Neurosci* 9:3728-3740.
- Mattson MP, Rychlik B (1990): Glia protect hippocampal neurons against excitatory amino acid induced degeneration: involvement of fibroblast growth factor. *Int J Dev Neurosci* 8:399-415.
- Matus A (1988): Microtubule-associated proteins: their potential role in determining neuronal morphology. *Ann Rev Neurosci* 11:29-44.
- Matus A, Bernhardt R, Bodner R, Alaimo-Beuret D (1986): Microtubule-associated protein 2 and tubulin are differently distributed in the dendrites of developing neurons. *Neurosci* 12:371-389.
- Mayford M, Barazilai A, Keller F, Schacher S, Kandel ER (1992): Modulation of an NCAM related adhesion molecule with long term synaptic plasticity in *Aplysia* *Science* 256:638-644.
- McCarthy KD, de Vellis J (1980): Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 85:890-902.
- McConnell P, Berry M (1982): Regeneration of ganglion cell axons in the adult mouse retina. *Brain Res* 241:362-365.
- McConnell SK (1991): The generation of neuronal diversity in the central nervous system. *Ann Rev Neurosci* 14:269-300.

- McConnell SK (1992): The control of neuronal identity in the developing cerebral cortex. *Curr Opin Neurobiol* 2:23-27.
- McEwan BS (1991): The role of hormones as mediators of a changing brain. *Proceedings of the Course on Developmental Neurobiology* 1:96-108.
- McKee AC, Kowall NW, Kosik KS (1989): Microtubular reorganization and dendritic growth response in Alzheimer's disease. *Ann Neurol* 26:652-659.
- McKeon RJ, Schreiber RC, Rudge JS, Silver J (1991): Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J Neurosci* 11:3398-3411.
- McMillian MK, Thai L, Hong JS, O'Callaghan JP, Pennypacker KR (1994): Brain injury in a dish: a model for reactive gliosis. *TINS* 17:138-142.
- Meiners S, Marone M, Rittenhouse JL, Geller HM (1993): Regulation of astrocytic tenascin by basic fibroblast growth factor. *Dev Biol* 160:480-493.
- Midtgaard J (1994): Processing of information from different sources: spatial synaptic integration in the dendrites of vertebrate CNS neurons. *TINS* 17:166-173.
- Miller MW (1988): Development of projection and local circuit neurons in the neocortex. In Peters A, Jones EG (eds): "Cerebral Cortex. Vol. 7. The Development and Maturation of the Cerebral Cortex." New York: Plenum Press pp 133-175.
- Miller RH, French-Constant C, Raff MC (1989): The macroglial cells of the rat optic nerve. *Annu Rev Neurosci* 12:517-534.
- Misson JP, Takahashi T, Caviness VS (1991): Ontogeny of radial and other Astroglial cells in murine cerebral cortex. *Glia* 4:138-148.
- Montoro RJ, Díaz-Nido J, Avila J, López-Barneo J (1993): N-methyl-D-aspartate stimulates the dephosphorylation of the microtubule-associated protein 2 and potentiates excitatory synaptic pathways in the rat hippocampus. *Neuroscience* 54:859-871.
- Moore IE, Bountempo JM, Weller RO (1987): Response of fetal and neonatal rat brain to injury. *Neuropathol Appl Neurobiol* 13:219-228.
- Moreno-Flores MT, Bovolenta P, Nieto-Sampedro M (1993): Polymorphonuclear leukocytes in brain parenchyma after injury and their interaction with purified astrocytes in culture. *Glia* 7:146-157.
- Morrison RS, de Vellis J (1981): Growth of purified astrocytes in a chemically defined medium. *Proc Natl Acad Sci USA* 78:7205-7209.
- Morrison RS, Kornblum HI, Leslie FM, Bradshaw RA (1987): Trophic stimulation of cultured neurons from neonatal rat brain by epidermal growth factor. *Science* 238:72-75.
- Morrison RS, Sharma A, deVellis J, Bradshaw RA (1986): Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. *Proc Natl Acad Sci USA* 83:7537-7541.

- Moumdjian RA, Antel JP, Yong VW (1991): Origin of contralateral reactive gliosis in the surgically injured rat cerebral cortex. *Brain Res* 547:223-228.
- Moya KL, Benowitz LI, Schneider GE, Allinquant B (1994): The amyloid precursor protein is developmentally regulated and correlated with synaptogenesis. *Dev Biol* 161:597-603.
- Mucke L, Oldstone MBA, Morris JC, Nerneberg MI (1991): Rapid activation of astrocyte-specific expression of GFAP-lacZ transgene by focal injury. *New Biol* 3:465-474.
- Murphy S, Minor RL, Welk G, Harrison DG (1990): Evidence for an astrocyte derived vasorelaxing factor with properties similar to nitric oxide. *J Neurochem* 55:349-351.
- Murphy S, Pearce B (1987): Functional receptors for neurotransmitters on astroglial cells. *Neuroscience* 22:381-394.
- Murphy S, Simmons ML, Agullo L, Garcia A, Feinstein DL, Galea E, Reid DJ, Minc-Golomb D, Schwartz JP (1993): Synthesis of nitric oxide in CNS glial cells. *TINS* 16:323-328.
- Nauta WJ, Freitag M (1986): "Fundamental Neuroanatomy." New York: WH Freeman, p 33.
- Neale EA, Bowers LM, Smith TG Jr (1993): Early dendrite development in spinal cord cell cultures: a quantitative study. *J Neurosci Res* 34:54-66.
- Neugebauer KM, Tomaselli KJ, Lilien J, Reichardt LF (1988): N-Cadherin, NCAM and integrins promote retinal neurite outgrowth on astrocytes *in vitro*. *J Cell Biol* 107:1177-1187.
- Nieto-Sampedro M, Gómez-Pinilla F, Knauer DJ, Broderick JT (1988): Epidermal growth factor receptor immunoreactivity in rat brain astrocytes. Response to injury. *Neurosci Lett* 91:276-282.
- Nieto-Sampedro M, Manthorpe M, Barbin G, Varon S, Cotman CW (1983): Injury-induced neuronotrophic activity in adult rat brain: correlation with survival of delayed implants in the wound cavity. *J Neurosci* 3:2219-2229.
- Noble M, Fok-Seang J, Cohen J (1984): Glia are a unique substrate for the *in vitro* growth of central nervous system neurons. *J Neurosci* 4:1892-1903.
- Norenberg MD (1979): The distribution of glutamine synthetase in the rat central nervous system. *J Histochem Cytochem* 27:756-762.
- Norris CR, Kalil K (1991): Guidance of callosal axons by radial glia in the developing cerebral cortex. *J Neurosci* 11:3481-3492.
- Norton WT, Farooq M (1989): Astrocytes cultured from mature brain derive from glial precursor cells. *J Neurosci* 9:769-775.
- Norton WT, Farooq M, Chiu F, Bettenstein JE (1988): Pure astrocyte cultures derived from cells isolated from mature brain. *Glia* 1:403-414.
- Noske W, Lentzen H, Herken H (1982): Phagocytosis and morphological development of rat astrocytes in primary cultures. *Cell Mol Biol* 28:235-244.
- O'Malley EK, Sieber B-A, Black IB, Dreyfus CF (1992): Mesencephalic type I astrocytes mediate the survival of substantia nigra dopaminergic neurons in culture. *Brain Res* 582:65-70.

- Orita T, Akimura T, Nishikazi T, Kamiryo T, Ikeyama Y, Aoki H, Ito H (1990): Transferrin receptors in injured brain. *Acta Neuropath Berlin* 79:686-688.
- Orkand RK (1977): Glial cells. In Brookhart JM, Mountcastle VB: "Handbook of Physiology. Section 1. The Nervous System." Baltimore: Williams and Wilkins, pp 855-875.
- Ortega A, Eshar N, Teichberg VI (1991): Properties of kainate receptor/channels on cultured Bergman glia. *Neuroscience* 41:335-349.
- Pachter J S, Liem RKH (1984): The differential appearance of neurofilament triplet proteins in the developing rat optic nerve. *Dev Biol* 103:200-210.
- Paulson OB, Newman EA (1987): Does the release of potassium from astrocyte endfeet regulate cerebral blood flow. *Science* 237:896-898.
- Pearce B, Murphy S, Jeremy J, Morrow C, Dandona P (1989): ATP evoked calcium mobilization and prostanoid release from astrocytes: P2 purinergic receptors linked to phosphoinositide hydrolysis. *J Neurochem* 52:971-977.
- Pennypacker K, Fischer I, Levitt P (1991): Early *in vitro* genesis and differentiation of axons and dendrites by hippocampal neurons analyzed quantitatively with neurofilament-H and microtubule-associated protein 2 antibodies. *Exp Neurol* 111:25-35.
- Perry G (1992): Alzheimer's disease. In Smith B, Adelman A (eds): "Neuroscience Year, Suppl 2." Boston: Birkhauser, pp 5-8.
- Perry VH Linden R (1982): Evidence for dendritic competition in the developing retina. *Nature* 297:683-685.
- Peters A, Palay SL, Webster H (1991): "The Fine Structure of the Nervous System: Neurons and Their Supporting Cells. 3rd edition." New York: Oxford University Press.
- Petit TL, Alfano DP, LeBoutillier JC (1983): Early lead exposure and the hippocampus: a review and recent advances. *Neurotoxicology* 4:79-94.
- Petit TL, LeBoutillier JC, Gregorio A, Libstug H.(1988): The pattern of dendritic development in the cerebral cortex of the rat. *Dev Brain Res* 41:209-219.
- Petito CK, Juurlink BHJ, Hertz L (1991): In vitro models differentiating direct and indirect effects of ischemia on astrocytes. *Exp Neurol* 113:364-372.
- Pike CJ, Cummings BJ, Cotman CW (1992): b-Amyloid induces neuritic dystrophy *in vitro*: similarities with Alzheimer pathology. *NeuroReport* 3:769-772.
- Pittack C, Jones M, Reh TA (1991): Basic fibroblast growth factor induces retinal pigment epithelium to generate neural retina *in vitro* . *Development* 113:577-588.
- Pollerberg G, Schachner M, Davoust J (1986): Differentiation state-dependent surface mobilities of two forms of neural cell adhesion molecule. *Nature* 324:462-465.
- Pomeroy SL, Purves D (1988): Neuron/glia relationships observed over intervals of several months in living mice. *J Cell Biol* 107:1167-1175.

- Pope A (1978): Neuroglia: Quantitative aspects. In: Schoffeniels E, Franck G, Hertz L, Tower DB (eds): "Dynamic Properties of Glia Cells." New York: Pergamon Press, pp 13-20.
- Popov VO, Bocharova LS, Bragin AG (1992): Repeated changes of dendritic morphology in the hippocampus of ground squirrels in the course of hibernation. *Neuroscience* 48:45-51.
- Povlishock JT, Erb DE, Astruc J (1992): Axonal response to traumatic brain injury: reactive axonal change, deafferentation, and neuroplasticity. *J Neurotrauma* 9(suppl):S189-S200.
- Price J, Hynes RO (1985): Astrocytes in culture synthesize and secrete a variant form of fibronectin. *J Neurosci* 5:2205-2211.
- Pringle NP, Mudhar HS, Collarini EJ, Richardson WD (1992): PDGF receptors in the rat CNS: during late neurogenesis, PDGF alpha-receptor expression appears to be restricted to glial cells of the oligodendrocyte lineage. *Development* 115:535-551.
- Prochiantz A, Delacourte A, Daguet M-C, Paulin D (1982): Intermediate filament proteins in mouse brain cells cultured in the presence or absence of fetal calf serum. *Exp Cell Res* 139:404-410.
- Prochiantz A, Joliot AH, Volovitch M (1993): Perspectives on the autocrine and paracrine functions of trans-activating factors. *Res Neurol Neurosci* 5:3-7.
- Prochiantz A, Rousset A, Chamak B (1990): Adhesion and the *in vitro* development of axons and dendrites. *Prog Brain Res* 86:331-336.
- Purpura DP (1975): Dendritic differentiation in human cerebral cortex: normal and aberrant developmental patterns. In: Kretzberg GW (ed): "Advances in Neurology." New York: Raven Press, pp 91-116.
- Purves D, Hadley TD, Voyvodic HT (1986): Dynamic changes in the dendritic geometry of individual neurons visualized over periods of up to three months in the superior cervical ganglion of living mice. *J Neurosci* 6:1051-1060.
- Purves D, Lichtman JW (1985): Principles of neural development. Sinauer, Sunderland, MA.
- Purves D, Snider WD, Voyvodic JT (1988): Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature* 336:123-128.
- Qian J, Bull MS, Levitt P (1992): Target-derived astroglia regulate axonal outgrowth in a region-specific manner. *Dev Biol* 149:278-294.
- Quon D, Want Y, Catalano R, Marian Scardina J, Murakami K, Cordell B (1991): Formation of B-amyloid protein deposits in brains of transgenic mice. *Nature* 352:239-241.
- Rable-Rückard H (1890): Sind die ganglianzellen amoboid? Eine hypothese zur mechanik psychischer vorgänge. *Neurol Centralbl Leipzig* 9:199.
- Raff MC (1989): Glial cell diversification in the rat optic nerve. *Science* 243:1450-1455.
- Raff MC, Lillien L, Richardson W, Burne J, Noble M (1988): Platelet derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* 333:562-565.

- Raff MC, Miller RH, Noble M (1983): A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* 303:390-396.
- Raff MC, Mirsky R, Fields KL, Lisak RP, Dorfman SH, Silberbeg DH, Gregson NA, Liebowitz S, Kennedy MC (1978): Galactocerebroside is a specific cell surface antigenic marker for oligodendrocytes in culture. *Nature* 274:813-876.
- Raisman G (1969): Neuronal plasticity in the septal nuclei of the adult rat. *Brain Res* 14:25-48.
- Rakic P (1988): Specification of cerebral cortical areas. *Science* 241: 70-175.
- Rakic P, Sidman RL (1973): Organization of cerebellar cortex secondary to deficit of granule cells in weaver mutant mice. *J Comp Neurol* 152:133-162.
- Ramoa AS, Campbell G, Schatz CJ (1988): Dendritic growth and remodeling of cat retinal ganglion cells during fetal and postnatal development. *J Neurosci* 8:4239-4261.
- Ramon y Cajal S (1894): Les nouvelles idées sur la structure du système nerveux chez l'homme et chez les vertébrés. Paris, Reinwald.
- Ramon y Cajal S (1954): Neuron theory or reticular theory? Objective evidence of the anatomical unity of nerve cells. Purkiss MU, Fox CA (trans): Madrid, Consejo Superior de Investigaciones Científicas Instituto Ramon y Cajal.
- Rapraeger A, Jalkanen M, Bernfield M (1986): Cell surface proteoglycans associate with the cytoskeleton at the basolateral surface of mouse mammary epithelial cells. *J Cell Biol* 103:2683-2696.
- Rees S, Harding R (1988): The effects of intrauterine growth retardation on the development of the Purkinje cell dendritic tree in the cerebellar cortex of fetal sheep: a note on the ontogeny of the Purkinje cell. *Int J Dev Neurosci* 6:461-469.
- Reh TA (1992): Cellular interactions determine neuronal phenotypes in rodent retinal cultures. *J Neurobiol* 23:1067-1083.
- Reichardt LF, Tomaselli KJ (1991): Extracellular matrix molecules and their receptors: functions in neural development. *Ann Rev Neurosci* 14:531-70.
- Reier PJ, Anderson DK, Thompson FJ, Stokes BT (1992): Neural tissue transplantation and CNS trauma: anatomical and functional repair of the injured spinal cord. *J Neurotrauma* 9(suppl):S223-S248.
- Reier PJ, Stensaas LJ, Guth L (1983): The astrocytic scar as an impediment to regeneration in the central nervous system. In: Kao CC, Bunge RP, Reier PJ (eds): "Spinal Cord Reconstruction." New York: Raven Press, pp 163-195.
- Renfranz PJ, Cunningham MG, McKay RDG (1991): Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* 66:713-729.
- Reynolds BA, Weiss S (1992): Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* :1707-1710.

- Richardson WD, Pringle N, Mosley MJ, Westermak B, DuBois-Dalcq M (1988): A role for platelet derived growth factor in normal gliogenesis in the central nervous system. *Cell* 53:309-319.
- Rio-Hortega P (1932): Microglia, In: Penfield W (ed) *Cytology and cellular pathology of the nervous system*. Hocker, New York, pp 481-584.
- Rogowski BA, Feng A (1982): Normal postnatal development of the medial superior olivary nucleus in the albino rat: a golgi and Nissl study. *J Comp Neurol* 207:369-380.
- Role LW, Fishbach GD (1987): Changes in the number of chick ciliary ganglion neuron processes with time in cell culture. *J Cell Biol* 104:363-370.
- Rosen CL, Bunge RP, Ard MD, Wood PM (1989): Type 1 astrocytes inhibit myelination by adult rat oligodendrocytes *in vitro*. *J Neurosci* 9:3371-3379.
- Rosenberg PA (1991): Accumulation of extracellular glutamate and neuronal death in astrocyte-poor cortical cultures exposed to glutamine. *Glia* 4:91-100.
- Rosenberg PA, Amin S, Leitner M (1992): Glutamate uptake disguises neurotoxic potency of glutamate agonists in cerebral cortex in dissociated cell culture. *J Neurosci* 12:56-61.
- Rossino P, Gavazzi I, Timpl R, Aumailley M, Abbadini M, Giancotti F, Silengo L, Marchisio PC, Tarone G (1990): Nerve growth factor induces increased expression of a laminin binding integrin in rat pheochromocytoma PC12 cells. *Exp Cell Res* 189:100-108.
- Rotwein P, Burgess SK, Milbrandt JD, Krause JE (1988): Differential expression of insulin-like growth factor genes in rat central nervous system. *Proc Natl Acad Sci USA* 85:265-269.
- Rouget M, Araud D, Seite R, Prochiantz A, Autillo-Touati A (1993): Astrocyte regulated synaptogenesis: an *in vitro* ultrastructural analysis. *Neurosci Lett* 150:85-88.
- Rousselet A, Autillo-Touati A, Araud D, Prochiantz A (1990): *In vitro* regulation of neuronal morphogenesis and polarity by astrocyte-derived factors. *Dev Biol* 137:33-45.
- Rousselet A, Fetler L, Chamak B, Prochiantz A (1988): Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia. *Dev Biol* 129:495-504.
- Rubel EW, Smith ZDJ, Steward O (1981): Sprouting in the avian brainstem auditory pathway: dependence on dendritic integrity. *J Comp Neurol* 202:397-414.
- Rudge JS, Silver J (1990): Inhibition of neurite outgrowth on astroglial scars *in vitro*. *J Neurosci* 10:3594-3603.
- Rudge JS, Smith GM, Silver J (1989): An *in vitro* model of wound healing in the CNS: analysis of cell reaction and interaction at different ages. *Exp Neurol* 103:1-16.
- Ruoslahti E, Yamaguchi Y (1991): Proteoglycans as modulators of growth factor activities. *Cell* 64:867-869.
- Rutka JT, Apodaca G, Stern R, Rosenblum M (1988): The extracellular matrix of the central and peripheral nervous system: structure and function. *J Neurosurg* 69:155-170.

- Saad B, Constam DB, Ortmann R, Moos M, Fontana A, Schachner M (1991): Astrocyte-derived TGF- β 2 and NGF differentially regulate neural recognition molecule expression by cultured astrocytes. *J Cell Biol* 115:473-484.
- Sanes DH, Goldstein NA, Ostad M, Hillman DE (1990): Dendritic morphology of central auditory neurons correlates with their tonotopic position. *J Comp Neurol* 294:443-454.
- Sanes DH, Markowitz S, Bernstein J, Wardlow J (1992a): The influence of inhibitory afferents of the development of postsynaptic dendritic arbors. *J Comp Neurol* 321:637-644.
- Sanes DH, Siverls V (1991): Development and specificity of inhibitory terminal arborizations in the central nervous system. *J Neurobiol* 22:837-854.
- Sanes DH, Song J, Tyson J (1992b): Refinement of dendritic arbors along the tonotopic axis of the gerbil lateral superior olive. *Brain Res* 67:47-55.
- Sanes JR (1989): Extracellular matrix molecules that influence neural development. *Ann Rev Neurosci* 12:491-516.
- Sargent PB (1989): What distinguishes axons from dendrites? Neurons know more than we do. *TINS* 12:6.
- Sarthy PV (1985): Establishment of Müller cell cultures from adult rat retina. *Brain Res* 337:138-141.
- Savio T, Schwab ME (1989): Rat CNS white matter but not gray matter is nonpermissive for neural cell adhesion and fiber outgrowth. *J. Neurosci* 9: 1126-1133.
- Savio T, Schwab ME (1990): Lesioned corticospinal tract axons regenerate in myelin free rat spinal cord. *Proc Natl. Acad. Sci USA* 87:4130-4133.
- Schacher S, Kandel ER, Montarola PG (1993): cAMP and arachidonic acid simulate long-term structural and functional changes produced by neurotransmitters in aplysia sensory neuron. *Neuron* 10:1079-1088.
- Schade JP, Meeter K, van Groenigen WB (1962): Maturational aspects of the dendrites of the human cerebral cortex. *Acta Morphol Neerl Scand* 5:37-48.
- Schilling K, Dickinson MH, Connor JA, Morgan JI (1991): Electrical activity in cerebellar cultures determines Purkinje cell dendritic growth patterns. *Neuron* 7:891-902.
- Schlaggar BL, O'Leary DDM (1991): Potential of visual cortex to develop an array of functional units unique to somatosensory cortex. *Science* 252:1556-1560.
- Schnell L, Schwab ME (1990): Axonal regeneration in the rat spinal cord produced by an antibody against myelin associated neurite growth inhibitors. *Nature* 343:269-272.
- Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME (1993): Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature* 367:170-173.
- Schousboe A (1981): Transport and metabolism of glutamate and GABA in neuron and glial cells. *Int Rev Neurobiol* 22:1-45.

- Schwab ME, Caroni P (1988): Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading *in vitro*. J Neurosci 8:2381-2393.
- Schwartz JP, Wilson DJ (1992): Preparation and characterization of type 1 astrocytes cultured from adult rat cortex, cerebellum, and striatum. Glia 5:75-80.
- Schwartz R, Whetsell WO, Mangano RM (1983): Quinolinic acid: An endogenous metabolite that produces axon-sparing lesions in the rat cortex. Science 229:315-318.
- Schwarz EA (1993): L-Glutamate conditionally modulates the potassium current of Müller glial cells. Neuron 10:1141-1149.
- Schwarz JP, Marini AM (1992): Regional specificity of astrocytes. In: Smith B, Adelman B (eds): "Neuroscience Year." Boston: Birkhauser, pp 135-136.
- Seil FJ, Eckenstein FP, Reier PJ (1992): Induction of dendritic spine proliferation by an astrocyte secreted factor. Exp Neurol 117:85-89.
- Seilheimer B, Schachner M (1987): Regulation of neural cell adhesion molecule expression on cultured mouse Schwann cells by nerve growth factor. EMBO 6:1611-1616.
- Shaw G, Banker GA, Weber K (1985): An immunofluorescence study of neurofilament protein expression by developing hippocampal neurons in tissue culture. Eur J Cell Biol 39:205-216.
- Shaw G, Weber K (1982): Differential expression of neurofilament triplet in brain development. Nature 298:277-279.
- Shepard SR, Ghajar JBG, Giannuzzi R, Kupferman S, Hariri RJ (1991): Fluid percussion barotrauma chamber: a new *in vitro* model for traumatic brain injury. J Surg Res 51:417-424.
- Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsburn M (1984): Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science 223:1296-1299.
- Shigeno T, Mima T, Takakura K, Graham D, Kato G, Hashimoto Y, Furukawa S. (1991): Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. J. Neurosci 11: 2914-2919
- Shinoda H, Marini AM, Cosi C, Schwartz JP (1989): Brain region and gene specificity of neuropeptide gene expression in cultured astrocytes. Science 245:415-417.
- Shoukimas GM, Hinds JW (1978): The development of the cerebral cortex in the embryonic mouse: an electron microscopic serial section analysis. J Comp Neurol 179:795-830.
- Siesjö BK (1992): Pathophysiology and treatment of focal cerebral ischemia. Part II: Mechanisms of damage and treatment. J Neurosurg 77:337-354.
- Silver J, Lorenz SE, Wahlsten D, Coughlin J (1982): Axonal guidance during development of the great cerebral commissures: descriptive and experimental studies, *in vivo*, on the role of preformed glial pathways. J Comp Neurol 210:10-29.
- Silver J, Ogawa MY (1983): Postnatally induced formation of the corpus callosum in acallosal mice on glia coated cellulose bridges. Science 220:1067-1069.

- Simeone A, Gulisano M, Acampora D, Stornaiulo A, Rambaldi M, Boncinelli E (1992): Two vertebrate homeobox genes related to drosophila empty spiracles genes are expressed in the embryonic cerebral cortex. *EMBO J* 11:2541-2550.
- Simons K, Dupree P, Fiedler K, Huber LA, Kobayashi T, Kurzchalia T, Olkkonen V, Pimplikar S, Parton R, Dotti C. (1992): Biogenesis of cell-surface polarity in epithelial cells and neurons. *Cold Spring Harb Symp Quant Biol* 57:611-619.
- Skoff RP, Knapp PE (1991): Division of astroblasts and oligodendroblasts in postnatal rodent brain: evidence for separate astrocyte and oligodendrocyte lineages. *Glia* 4:165-174.
- Sladek JR, Gash DM (1988): Nerve cell grafting in Parkinson's disease. *J Neurosurg* 68:337-351.
- Smith CL (1994): Cytoskeletal movements and substrate interactions during initiation of neurite outgrowth by sympathetic neurons *in vitro*. *J Neurosci* 14:384-398.
- Smith GM, Miller RH, Silver J (1986): Changing role of forebrain astrocytes during development, regenerative failure and induced regeneration upon transplantation. *J Comp Neurol* 251:23-43.
- Smith GM, Rutishauser Y, Silver J, Miller RH (1990): Maturation of astrocytes *in vitro* alters the extent and molecular basis of neurite outgrowth. *Dev Biol* 138:377-390.
- Smith ZDJ (1981): Organization and development of brain stem auditory nuclei of the chicken: dendritic development in N. Laminaris. *J Comp Neurol* 203:309-333.
- Smithson KG, Suarez I, Hatton GI (1990): Beta-adrenergic stimulation decreases glial and increases neural contact with the basal lamina in rat intermediate lobes incubated *in vitro*. *J Neuroendocrinol* 2:693-699.
- Snider WD (1988): Nerve growth factor enhances arborization of sympathetic ganglion cells in developing mammals. *J Neurosci* 8:2628-2634.
- Snider WD, Zhang L, Yusoof S, Gorukanti N, Tsering C (1992): Interactions between dorsal root axons and their target motor neurons in developing mammalian spinal cord. *J Neurosci* 12:3494-3508.
- Snow AD, Wight TN (1989): Proteoglycans in the pathogenesis of Alzheimer's disease and other amyloidoses. *Neurobiol Aging* 10:481-497.
- Snow DM, Lemmon V, Cavrino DA, Caplan AI, Silver J (1990a): Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*. *Exp Neurol* 109:11-130.
- Snow DM, Steindler DA, Silver J (1990b): Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. *Dev Biol* 138:359-376.
- Sola C, Garcia-Ladona FJ, Sarasa M, Mengod G, Probst A, Palancios G, Palacios JM (1993): b-amyloid precursor protein gene expression is increased in the rat brain after motor neuron axotomy. *Eur J Neurosci* 5:795-808.
- Solomon F (1992): Neuronal cytoskeleton and growth. *Curr Opin Neurobiol* 2:613-617.

- Sontheimer H, Waxman SG, Ransom BR (1991): Relationship between sodium current expression and cell-cell coupling in astrocytes cultured from rat hippocampus. *J Neurophysiol* 65:3-19.
- Spillantini MG, Goedert M, Jakes R, Klug A (1990): Topographical relationship between B-amyloid and tau-protein epitopes in tangle-bearing cells in Alzheimer's disease. *Proc Natl Acad Sci USA* 87:3952-3956.
- Spruston N, Jaffe DB, Johnston D (1994): Dendritic attenuation of synaptic potentials and currents: the role of passive membrane potentials. *TINS* 17:161-166.
- Steindler DA (1993): Glial boundaries in the developing nervous system. *Annu Rev Neurosci* 16:445-470.
- Steindler DA, Cooper NGF, Faissner A, Schachner M (1989): Boundaries defined by adhesion molecules during development of the cerebral cortex: The J¹/Tenascin glycoprotein in the mouse somatosensory cortical barrel field. *Dev Biol* 131:243-260.
- Sternberger L, Sternberger N (1983): Monoclonal antibodies distinguish phosphorylated and dephosphorylated forms on neurofilaments in situ. *Proc Natl Acad Sci USA* 80:6126-6130.
- Steward O (1989a): "Principles of Cellular, Molecular and Developmental Neuroscience." New York: Springer-Verlag.
- Steward O (1989b): Reorganization of neuronal connections following CNS trauma: principles and experimental paradigms. *J Neurotrauma* 6:99-152.
- Steward O, Falk PM (1986): Protein-synthetic machinery at postsynaptic sites during synaptogenesis: a quantitative study of the association between polyribosomes and developing synapses. *J Neurosci* 6:412-423.
- Steward O, Torre ER, Phillips LL, Trimmer PA (1990): The process of reinnervation in the dentate gyrus of adult rats: time course of increases in mRNA for glial fibrillary acidic protein. *J Neurosci* 10:2373-2384.
- Strong MJ, Svedmyr A, Gajdusek DC, Garruto RM (1990): The temporal expression of amyloid precursor protein mRNA *in vitro* in dissociated hippocampal neuron cultures. *Exp Neurol* 109:171-179.
- Sugiyama K, Brunori A, Mayer ML (1989): Glial uptake of excitatory amino acids influences neuronal survival in cultures of mouse hippocampus. *Neuroscience* 32:779-791.
- Sutton JK, Brunso-Bechtold JK (1993): Dendritic development in the dorsal lateral geniculate nucleus of ferrets in the postnatal absence of retinal input: a golgi study. *J Neurobiol* 24:317-334.
- Svendsen CN, Kew JNC, Staley K, Sofroniew MV (1994): Death of developing septal cholinergic neurons following NGF withdrawal *in vitro*: protection by protein synthesis inhibition. *J Neurosci* 14:75-87.
- Taft WC, Yang K, Dixon CE, Hayes RL (1992): Microtubule-associated protein 2 levels decrease in hippocampus following traumatic brain injury. *J Neurotrauma* 9:281-290.

- Takeichi M (1988): The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* 102:639-655.
- Tecoma ES, Monyer H, Goldberg MP, Choi DW (1989): Traumatic neuronal injury *in vitro* is attenuated by NMDA antagonists. *Neuron* 2:1541-1545.
- Terry RD, Katzman R (1983): Senile dementia of the Alzheimer type. *Ann Neurol* 14:497-501.
- Thanos S (1991): The relationship of microglial cells to dying neurons during natural neuronal cell death and axotomy induced degeneration. *Eur J Neurosci* 1:19-26.
- Thanos S, Mey J, Wild M (1993): Treatment of the adult retina with microglia-suppressing factors retards axotomy-induced neuronal degradation and enhances axonal regeneration *in vivo* and *in vitro*. *J Neurosci* 13:455-466.
- Theodosis DT, Poulain DA (1989): Neuronal-glial and synaptic plasticity in the adult rat paraventricular nucleus. *Brain Res* 484:361-366.
- Thiery JP, Boyer B (1992): The junction between cytokines and cell adhesion. *Curr Opin Cell Biol* 4:782-792.
- Thiery JP, Delouree A, Grumet M, Edelman GM (1985): Initial appearance and regional distribution of the neuron-glia cell adhesion molecule. *J Cell Biol* 100:442-456.
- Tomaselli K, Neugebauer KM, Bixby JL, Liliei J, Reichardt LF (1988): N-cadherin and integrins: Two receptor systems that mediate neuronal process outgrowth on astrocyte surfaces. *Neuron* 1:33-43.
- Tomaselli KJ, Doherty P, Emmett CJ, Damsky CH, Walsh FS, Reichardt LF (1993): Expression of B1 integrins in sensory neurons of the dorsal root ganglion and their functions in neurite outgrowth on two laminin isoforms. *J Neurosci* 13:4880-4888.
- Tomaselli KJ, Reichardt LF, Bixby JL (1986): Distinct molecular interactions mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. *J Cell Biol* 103:2659-2672.
- Topp KS, Faddis BT, Vijayan VK (1989): Trauma-induced proliferation of astrocytes in the brains of young and aged rats. *Glia* 2:201-211.
- Torre ER, Steward I (1992): Demonstration of local protein synthesis within dendrites using a new cell culture system that permits the isolation of living axons and dendrites from their cell bodies. *J Neurosci* 12:762-772.
- Trimmer PA, Wunderlich RE (1990): Changes in astroglial scar formation in rat optic nerve as a function of development. *J Comp Neurol* 296:359-378.
- Tropea M, Johnson MI, Higgins D (1988): Glial cells promote dendritic development in rat sympathetic neurons *in vitro*. *Glia* 1:380-392.
- Tsacopoulos M, Evequoz-Mercier V, Perrottet P, Buchner E (1988): Honeybee retinal glial cells transform glucose and supply the neurons with metabolic substrate. *Proc Natl Acad Sci USA* 85:8727-8731.

- Tsacopoulos M, Veuthy AL, Saravelos SG, Perrottet P, Tsoupras G (1994): Glial cells transform glucose to alanine which fuels the neurons in the honeybee retina. *J Neurosci* 14:1339-1351.
- Tsuyama S, Terayama Y, Matsuyama S (1987): Numerous phosphates of microtubule associated protein 2 in living rat brain. *J Biol Chem* 262:10886-10892.
- Twitty VC, Johnson HH (1934): Motor inhibition in *Amblystoma* produced by *Triturus* transplants. *Science* 80:78-79.
- Unsicker K, Reichert-Preisch H, Schmidt R, Pettmann B, Labourdette G, Sensenbrenner M (1987): Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous system neurons. *Proc Natl Acad Sci* 84:5459-5463.
- Valverde F (1968): Structural changes in the area striata of the mouse after enucleation. *Exp Brain Res* 5: 274-292.
- Van der Loos H (1965): The "improperly" oriented pyramidal cell in the cerebral cortex and its possible bearing on problems of growth and orientation. *Bull Johns Hopkins Hosp* 117:228-250.
- van den Pol AN, Finkbeiner SM, Cornell-Bell AH (1992): Calcium excitability and oscillations in suprachiasmatic nucleus neurons and glia *in vitro*. *J Neurosci* 12:2648-2664.
- Vaughan JE (1989): Fine structure of synaptogenesis in the vertebrate central nervous system. *Synapse* 3:255-285.
- Vescovi AL, Reynolds BA, Fraser DD, Weiss S (1993): bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (Neuronal/astroglia) EGF generated CNS progenitor cells. *Neuron* 11:951-966.
- Vickland H, Westrum LE, Kott JN, Patterson SL, Bothwell MA (1991): Nerve growth factor receptor expression in the young and adult rat olfactory system. *Brain Res* 565:269-279.
- Vige X, Costa E, Wise BC (1991): Mechanisms of nerve growth factor mRNA regulation by interleukin-1 and basic fibroblast growth factor in primary cultures of rat astrocytes. *Mol Pharmacol* 40:186-192.
- Vilbulsreth S, Hefti F, Ginsberg MD, Dietrich WD, Busto R (1987): Astrocytes protect cultured neurons from degeneration induced by anoxia. *Brain Res* 422:303-311.
- Villegas-Pérez MP, Vidal-Sanz M, Bray GM, Aguayo AJ (1988): Influences of peripheral nerve grafts on the survival and regrowth of axotomized retinal ganglion cells in adult rats. *J Neurosci* 8:265-280.
- Violette SM, Shashikant CS, Salbaum JM, Belting HG, Wang JC, Ruddle FH (1992): Repression of the b-amyloid gene in a Hox. 3.1-producing cell line. *Proc Natl Acad Sci USA* 89:3805-3809.
- Voyvodic JT (1987): Development and regulation of dendrites in the rat superior cervical ganglion. *J Neurosci* 7:904-912.
- Walicke P, Cowan WM, Ueno N, Baird A, Guillemin R (1986): Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc Natl Acad Sci USA* 83:3012-3016.

- Wang LC, Baird DH, Hatten ME, Mason CA (1994): Astroglial differentiation is required for support of neurite outgrowth. *J. Neurosci* 14:3195-3207.
- Weinstein DE, Shelanski ML, Liem RKH (1991): Suppression by antisense mRNA demonstrates a requirement for the glial fibrillary acidic protein in the formation of stable astrocytic processes in response to neurons. *J Cell Biol* 112:1205-1213.
- Weiss GM, Pysh JJ (1978): Evidence for loss of Purkinje cell dendrites during late development: a morphometric golgi analysis in the mouse. *Brain Res* 154:219-230.
- Wesselingh SL, Gough NM, Finlay-Jones JJ, McDonald PJ (1990): Detection of cytokine mRNA in astrocyte cultures using the polymerase chain reaction. *Lymphokine Research* 9:177-185.
- Wetmore C, Cao Y, Pettersson RF, Olson L (1991): Brain-derived neurotrophic factor: subcellular compartmentalization and interneuronal transfer as visualized with anti-peptide antibodies. *Proc Natl Acad Sci USA* 88:9843-9847.
- Wetmore C, Olson L, Bean AJ (1994): Regulation of brain-derived neurotrophic factor (BDNF) expression from hippocampal neurons is mediated by non-NMDA type glutamate receptor. *J Neurosci* 14:1688-1700.
- Williams BP, Abney ER, Raff MC (1985): Macroglial cell development in embryonic rat brain: studies using monoclonal antibodies, fluorescence activated cell sorting and cell culture. *Dev Biol* 112:126-134.
- Williams E, Furness J, Walsh FS, Doherty P (1994): Characterization of the second messenger pathway underlying neurite outgrowth stimulated by FGF. *Development* 120:1685-1693.
- Wise SP, Fleshman JW, Jones EG (1979): Maturation of pyramidal cell form in relation to developing afferent and efferent connections of rat somatic sensory cortex. *Neurosci* 4:1275-1297.
- Wong-Riley MT (1989): Cytochrome oxidase: An endogenous metabolic marker for neuronal activity. *TINS* 12:94-101.
- Woodward WR, Nishi R, Meshul CK, Williams TE, Coulombe M, Eckenstein FP (1992): Nuclear and cytoplasmic localization of basic fibroblast growth factor in astrocytes and CA2 hippocampal neurons. *J Neurosci* 12:142-152.
- Wu R, Barish ME (1994): Astroglial modulation of transient potassium current development in cultured mouse hippocampal neurons. *J Neurosci* 14:1677-1687.
- Yamada K, Kinoshita A, Kohmura E, Sakaguchi T, Taguchi J, Kataoka K, Hayakawa T (1991): Basic fibroblast growth factor prevents thalamic degeneration after cortical infarction. *J Cereb Blood Flow Metab* 11:472-478.
- Yamauchi T, Fujisawa H (1982): Phosphorylation of microtubule associated protein-2 by calmodulin dependent protein kinase which occurs only in brain tissue. *Biochem Biophys Res Commun* 109:975-981.
- Yamauchi T, Fujisawa H (1988): Regulation of the interaction of actin filaments with microtubule associated protein-2 by calmodulin dependent protein kinase II. *Biochem Biophys Acta* 968:77-85.

- Yawo H (1987): Changes in the dendritic geometry of mouse superior cervical ganglion cells following postganglionic anatomy. *J Neurosci* 7:3703-3711.
- Yayon A, Lagsbrun M, Esko JD, Leder P, Ornits DM (1991): Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 64:841-848.
- Yeh H-J, Ruit KG, Wang Y-X, Parks WC, Snider WD, Deuel TF (1991): PDGF A-chain gene is expressed by mammalian neurons during development and in maturity. *Cell* 64:209-216.
- Yoshida K, Gage F (1991): Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. *Brain Res* 538:118-126.
- Young SH and Poo MM (1983): Spontaneous release of transmitter from growth cones of embryonic neurons. *Nature* 305, 634-637.
- Yu ACH, Lee YL, Eng LF (1993): Astrogliosis in culture: I. The model and the effect of antisense oligonucleotides on glial fibrillary acidic protein synthesis. *J Neurosci Res* 34:295-303.
- Zafra F, Lindholm D, Castrén E, Hartikka J, Thoenen H (1992): Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J Neurosci* 12:4793-4799.
- Zahs KR, Bigornia V, Deschepper CF (1993): Characterization of "plasma proteins" secreted by cultured rat macroglial cells. *Glia* 7:121-133.
- Zhang H, Miller RH, Rutishauser U (1992): Polysialic acid is required for optimal growth of axons on a neuronal substrate. *J Neurosci* 12:3107-3114.